Modulation of Apoptosis by Endogenously Produced Nitric Oxide

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Table of contents

Abstract

Nitric oxide (NO) has many important roles in the human nervous, immune and vascular system, and it also plays crucial roles in tumorgenesis. Many human tumors express inducible NO synthetase (NOS2/iNOS); however the roles of NO in tumor development and in the immune system are not fully understood.

The role of nitric oxide (NO) in cells is controversial. Both cytotoxic and cyto-protective roles have been reported. The effects of endogenous inducible NO synthesis on apoptosis sensitivity have been attempted to be elucidated in this study, and apoptosis mediated by the CD95 or TRAIL system was given particular attention. The hypothesis tested was whether persistent exposure to NO induces apoptosis and can select for cells with reduced sensitivity to NO and apoptosis.

A NOS2 expression vector driven by an ecdysone-inducible promoter was constructed; the vector was then introduced into EcR293 cells and EcR293-NOS2 stable cells were established. Ponasterone A, an ecdysone analog, was used to induce NOS2 expression. The advantage of this system is that cell lines could be established without constant exposure to NO. The expression of NOS2 and NO products were analyzed by Western blot, immunofluoresence staining, and Griess assays. The influences of NO on CD95- and TRAIL-mediated apoptosis were determined through FACS analysis, cytotoxicity assays, Western blots, and caspase activity.

The expression of NOS2 and NO product (nitrite) were detectable in a number of cell clones after induction by ponasterone A and NOS2 expression was dependent on the dose of ponasterone A. As the dose of ponasterone A increased, apoptosis induced by treatment with agonistic CD95 antibody (anti-APO-1) or TRAIL, proportionally increased compared to controls. In CD95-mediated apoptosis, loss of mitochondria membrane potential, the activation of caspase-8, -9, and -3 were observed. The same results also were obtained from TRAIL-mediated apoptosis pathway. In EcR293-NOS2 cells, it was found that NO can reduce cell proliferation compared with uninduced cells. The cytotoxicity of NO may involve mitochondria depolarization and endoplasmic reticulum (ER) stress signalling pathway, because ER stress-associated

proteins, such CHOP, BiP and ER-associated caspase-4 activation, were enhanced under the expression of NOS2.

The effects of long-term exposure to NO were further examined. NO induction increased the apoptosis substantially over uninduced controls after nine days of culture. To test the hypothesis that persistent expression of NO selects for cells with reduced sensitivity to apoptosis, EcR293-NOS2 cells were cultured under long term NO induction. Significant cell death was observed in these cultures but cell colonies grew which were selected for further cultivation. After approximately three months of culturing in the presence of ponasterone A, cell clones were analyzed for NO production. All the selected colonies, which survived cultivation in the presence of high concentration of ponasterone A, also produced NO. These results demonstrated that the survival of the colonies was due to NO resistance and not merely to loss of ponasterone inducible NO production. To assess the effects on apoptosis, cells were treated with agonistic CD95 antibodies and analyzed by FACS. Interestingly, it was found that the NO resistant cell lines were cross resistant to CD95-mediated apoptosis and NO resistant cell lines showed a reduced sensitivity to CTL killing. These results support the idea that chronic exposure to NO selects cells with reduced sensitivity to apoptosis.

On the basis of these results, it is suggested that the apoptosis-enhancing effect of NO results in the elimination of cells with a fully functional apoptotic response and the retention of a subpopulation of cells with an aberrant or attenuated response to death inducing signals. Chronic exposure to NO thus facilitates the clonal evolution of a population of cells that can circumvent normal death inducing signals, including those derived from CTLs during immune surveillance. These results support a role for NO in accelerating tumor formation and progression.

1. Introduction

1.1 Tumorigenesis

In most organs and tissues of the human body, a balance is carefully maintained between cell renewal and cell death. The various types of mature cells in the body have a limited life span, as these cells die, new cells may be generated by the proliferation and differentiation of various types of stem cells. Under normal conditions the production of new cells is carefully controlled and regulated to keep constant the number of various types of cells. Uncontrolled growth of the cells from the organs or tissues can result in tumors. Malignant tumors, which develop from benign, have progressively invasive abilities, metastasis, finally leading to cancer (Cairas, 1975). Malignant tumors or cancers are classified according to the embryonic origin of the tissues from which the tumor is derived. Carcinomas are tumors, which derive from endodermal or ectodermal tissues, such as skin or the epithelial tissue of internal organs and glands. Most of cancers like breast, colon cancers, prostate and lung cancers are carcinomas. Leukemia is the malignant tumors of hematopoietic cells and lymphomas are also the tumor cells derived from hematopoietic cells. Sarcomas are derived from mesodermal tissues, such as bone and fat (Immunology, fifth edition).

1.1.1 Genes involved in cancers

Cancer-associated genes can be mainly divided into three categories: oncogenes, tumor suppressor genes and genes related with cell death. Cellular oncogenes, also called proto-oncogenes, are the normal gene products, which encode proteins, involved in the functions of material exchange, induced cellular proliferation and other functions in the cells. Through deletion or point mutation in the open reading frames, or gene amplification and chromosome rearrangements, proto-oncogenes become oncogenes which can have pathological consequences for the organism. Overexpression of any these oncogenes may lead to uncontrolled growth or abnormal proliferation of the cells (Immunology, fifth edition). While the mutation of the oncogenes are dominant, and the mutation of one allele will be enough for overexpression of the proteins or change of the function, ultimately leading to the activation of the oncogene products in the cells. Oncogenes maybe result in qualitative or quantitative differences of their

products in the cells compared to the normal status which may increase uncontrolled proliferation of the cells. These changes may enhance the susceptibility for the cells to develop into tumors.

Beside oncogenes the second category of cancer-associated genes are called tumor-suppressor genes or anti-oncogenes encoding the proteins that inhibit excessive proliferation of the cells. Homeostasis in normal tissue is maintained by a highly regulated process of cellular proliferation balanced by cell death. Both oncogenes and tumor suppressor genes play an important role in this homeostatic process by regulating the cell proliferation or cell death through complicated and multiplied steps signalling way.

Tumor suppressor genes were classified by Kinzler and Vogelstein into different groups, namely gatekeeper- and caretaker- gene (Kinzler, 1997). For example Retinoblastoma (Rb) gene belongs to the first group and under normal conditions Rb gene controls the process of cell cycles. Loss or mutation of Rb gene products will lead to uncontrolled cell division and finally may result into a tumor. However the second group of the tumor suppressor genes showed different properties, loss or mutation of gene product may not directly lead to a tumor, but it will enhance the possibility for the mutation rate of all genes. P53 is one of the most important tumor suppressor genes. The protein of p53 gene functions as the regulator of cell cycles. Under stress the tumor suppressor gene p53 will block the cell cycle for repairing of DNA damage. While loss of p53 gene product may lead to accumulation of mutations in the cells and finally trigger cells into tumor development. For example, over 90 % of small-cell lung cancers and over 50 % of breast and colon cancers have been shown to be associated with mutations in p53 gene. The caretaker gene group was also termed DNA repair-enzymes, the mutation of these genes will result in to genetic instability. Nearly 30 human tumor suppressor genes were already cloned (Knudson, 1998). Most of them are the components of signal transduction cascades involved in cell cycle, DNA repair or apoptosis pathway (Cox, 1995). Also it is reported that the accumulation of p53 will induce cell death (Polyak, 1997), and p53 induces cells into apoptosis through a three-step process: (1) the transcriptional induction of redox-related genes; (2) the formation

of reactive oxygen species; and (3) the oxidative degradation of mitochondrial components, culminating in cell death.

The third category of cancer-associated genes or factors is involved with regulating the signalling pathway of programmed cell death or apoptosis. These genes encode the proteins involved in apoptosis pathway and the products may block or induce cells to undergo apoptosis. For example, bcl-2, an anti-apoptosis gene, was shown to play a crucial role in regulating cell survival during hematopoiesis and in the survival of selected B cells and T cells during maturation. This anti-apoptosis gene may increase the survival rate of tumor cells under immune surveillance or clinical therapy. Bcl-2 reduces apoptosis sensitivity through interfering the release of cytochrome c from mitochondria and activation of caspase-3 (Park, 2001).

1.1.2 Immune system and cancer

Cancer develops from cells which have lost the growth restraints of normal cells; as these cells continue to divide, collections of abnormal cells accumulate. These cells infiltrate and invade surrounding normal tissues, where they continue to multiply and finally form tumors, this process of spreading to a distant site is called metastasis. In the body, the immune system plays a crucial role in the control of tumor cells. It has been suggested that cancer cells frequently arise in the body but are recognized as foreign antigens and identified and eliminated by the immune system. For example, cytotoxic T lymphocytes can recogenize and remove tumor cells through inducing apoptosis. According to these concepts, tumors develop only if the cancer cells are successfully able to escape from immune surveillance. The tumor cells can achieve this purpose through reducing their expression of tumor antigens, which can induce immune responses of eliminating the tumor cells. Namely the tumor has mechanisms of resistant to immune system-triggered elimination. CD95 receptor/ligand system plays important role in immune surveillance; CTLs can induce in tumor cells apoptosis through this CD95 receptor/ligand signalling pathway. Lack of the expression of CD95 receptor on the cell surface or through expression of CD95 ligand in tumor cells leads to resistance to CD95-mediated apoptosis. These mechanisms may be operated in many tumors, such as human pancreatic cancer cells, renal cell carcinomas, breast

carcinomas, gastric carcinomas and colon cancers, and help the tumor cell to escape surveillance by the immune system (Strand, 1998; Strand, et al., 1998; Hug, 1997; Nambu, 1998; von Bernstorff, 1999; Satoh, 1999; Kim, 2000; Elnemr, 2001; Bernstorff, 2002).

1.1.3 Apoptosis and cancer development

Under normal conditions, the organism needs a mechanism that controls the balance between cell proliferation and cell death, and for this purpose exists the mechanism, which not only renews cells but also deletes cells. The balance between the cell proliferation and death is finely turned and is important for physiological and biological functions of cells. Through apoptosis, excess cells during the embryonic development, tumor cells and cells infected by virus are eliminated.

Alteration in the apoptosis sensitivity is a well documented phenomenon contributing to the pathomechanism of a wide variety of diseases (Krammer, 1998; Galle, 1997). Modulation in the control of apoptosis mediated through the CD95 receptor/ligand system contributes to the pathogenesis for a number of disorders such as cancer, autoimmunity, AIDS, and liver disease (Dhein, 1995; Strand, 1996; Krammer, 1998; Strand 1998; Rudi, 19996; Galle, 1998; Kondo, 1997). Many studies have reported that reduced sensitivity to CD95-mediated apoptosis is common in cancer cells or loss of CD95-induced apoptosis is one important step in tumorigenesis. In many tumors, such as small cell lung cancer (Lee, 1999b), malignant melanoma (Shin, 1999), and blast carcinoma (Lee, 1999) showed that a somatic mutation for loss of CD95 expression were observed. Loss of CD95-mediated apoptosis may provide tumor cells with critical survival advantages and finally promote malignancy (Lee, 2003, Raisova, 2000; Los, 1997). Furthermore, the selection for resistance to apoptosis-inducing signalling may prevent tumor cells from elimination by immune surveillance. Cytotoxic T lymphocytes (CTLs) have been consistently implicated in tumor surveillance and regression. One system, which plays an effector role in immune surveillance, is CD95-mediated apoptosis of tumor cells by infiltrating T-lymphocytes. Through resistance to CD95-mediated apoptosis tumor cells escape from immune surveillance.

Furthermore, natural killer (NK) cells constitutively express TRAIL, this expression significantly contributes to the cytotoxic effects of activated NK cells. Disruption of TRAIL/TRAIL-R system may enhance the susceptibility of tumor development. Mutation of TRAIL-R1 and TRAIL-R2 reduces or inhibits sensitivity of B cells to TRAIL treatment, and the tumor cells expressing TRAIL induces T cell into apoptosis and through this possible way tumor cells suppress the immune surveillance. Some studies indicated that cancers such as those that are found in the brain, lung and breast, most often display allelic loss on chromosome 8p21-22, where four of the five TRAIL receptors were mapped. TRAIL-R1 and TRAIL-R2 mutations have been found in metastatic breast cancer, non-Hodgkin lymphomas, head and neck as well as non-small cell lung cancer. Therefore loss or reduction of TRAIL-mediated apoptotic sensitivity may be involved in tumor development (MacFarlane, 1997; Marsters, 1997; Lee, 2001; Shin, 2001).

1.2 Apoptosis

The word, apoptosis, comes from the Greek word „Apo“(implication of away from or separation from) and „ptosis“(to fall). From literal meaning apoptosis presents something falling away just like the leaves falling away from a tree. From the biological and physiological point apoptosis, also called programmed cell death, was originally termed for morphological alterations, but is now generally used to describe the evolutionary, highly conserved, biological process, which finally leads to the typical biochemical and morphological alterations in cells and result to cell death.

During apoptotic processes, the outer plasma membrane of the cells still retain their integrity while chromatin condensation, shrinkage of the cells, DNA degradation to small fragments and a variety of proteolytic activity cascades have been happened. And dead cells or their debris (apoptotic bodies) are rapidly phagocytosed by neighboring cells or macrophages before lysis occurs. This ensures that their intracellular contents, including proteolytic and other lytic enzymes, cationic proteins, and oxidizing molecules are not released into surrounding tissues. The organism removes the excess cells, tumor cells and dead cells through apoptosis without any forward inflammatory reaction. On the contrary, during necrosis, cell death different

from apoptosis, the cell and their organelles swell and rupture, finally the intracellular constituents are released which evoke a secondary inflammatory response. Typically the shrinkage of the nuclei and loss of the plasma membrane integrity are observed during necrosis process (Denecker, 2001).

1.2.1 Death receptors

Death receptors are a subset of the tumor necrosis factor receptor (TNFR) family, and they are capable of triggering apoptosis by engaging a chain of caspase activations. The death receptors normally contain an intracellular death domain (DD), which is important for transduction of the apoptosis signal. CD95- (also known as Fas and Apo-1) and TRAIL-receptors are two members of TNFR family, and both play crucial roles in physiological and pathological mechanism in regulating cell death (Lavrik, 2005).

1.2.1.1 CD95 receptor

CD95-mediated death pathway is activated through the receptor binding to its specific ligand (CD95L) or agonistic antibody (anti-APO-1), which then leads to cell death through activation of downstream signalling cascades. The CD95 receptor/ligand system plays a physiologically crucial role in T- and B-cell development and homeostasis. In activation induced cell death (AICD) process, after stimulation of TCR/CD3 complex on activated T- cells, in the absence of co-stimulated cytokine signal, CD95L is expressed and it triggers the CD95 receptor/ligand system in auto- and paracrine forms of apoptosis. Other studies showed also that activation-induced CD95L expression and the engagement of CD95 on the activated T cells were responsible for AICD in T-cell hybridomas and the Jurkat lymphoma (Lavrik, 2005; Brunner, 1995; Dhein, 1999). Other groups reported that CD95 and CD95L were necessary for AICD in mature T cells in vitro and in vivo. CD95 and CD95L contributed at least partially to peripheral deletion. This mechanism functioned as elimination and self-control in the immune response. Immune competent cells removed or eliminated the target cells through activating CD95-mediated apoptosis pathway, the physiological infected cells or nonself cells could be eliminated by discrimination through the MHC and TCR-complex (Frehch, 1999). Besides CD95 receptor/ligand system, perforin/Granzym B-system is also responsible for cytotoxic T cells function,

however some studies (Zaitsu, 2004; Shresta, 1997) showed that this system alone did not activate a full elimination affects in vivo. Furthermore, in the “immune-privilege tissue” there is another mechanism: these tissue expressed CD95L to remove T-cells. For example, in eyes, cellular immune response for associated inflammatory reaction is performed irreversibly.

CD95 is constitutively expressed in the liver and is shown to play an important role in pathological functions. Primary hepatocytes show a high apoptosis sensitivity after ex vivo stimulation with agonistic CD95-antibody (anti-APO-1) (Galle, 1995). Under pathophysiological conditions, for example in M. Wilson (Strand et al., 1998) disease, the alcoholic liver damage and viral hepatitis (Galle, 1994; Galle, 1998), showed a higher decline of hepatocytes, this was correlated with a high expression of CD95 receptor on the cells.

1.2.1.2 TRAIL receptors

TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), also termed as Apo-2 ligand, is a member of the TNF family of cytokines, which are structurally related proteins playing key roles in regulating cell death, inflammation and immune response in the cells. Like most other members of the TNF superfamily of ligands, Apo2L/TRAIL is primarily expressed as a type II membrane protein of 33–35 kD. As a cytotoxic molecule, it has been demonstrated that, compared with other members of the TNF family, TRAIL has the unique feature in the cytotoxicity, the ability to induce apoptosis in a number of tumor cells both in vitro and in vivo while displaying minimal cytotoxicity to the normal tissues or cells (Ashkenazi, 1999). The differential cytotoxicity of TRAIL in normal cells to tumor cells was proposed by the high concentration of decoy receptors in normal cells to protect these cells, including endothelial cells, from apoptosis (Gura, 1997; Dixit, 1999). The mRNA for Apo2L/TRAIL is detected in a wide variety of tissues including PBMC, spleen, thymus, prostate, ovary, small intestine, colon and placenta, but not the liver, testis or brain (Bouralexis, 2005).

Four human receptors specific for TRAIL have been already identified, including TRAIL-R1/DR4, TRAIL-R2/DR5, TRAIL-R3/DcR1 and TRAIL-R4/DcR2. All of these receptors belong to the apoptosis-inducing TRF-receptor (R) family. TRAIL-R1 and TRAIL-R2 both are type I trans-membrane protein, TRAIL-R1 contains 226 amino acid extra-cellular region, a 19 amino acid trans-membrane segments and a 220 amino acid cytoplasmic domain; and TRAIL-R2 have 411 amino acids including a 132 amino acid extra-cellular region, a 22 amino acid trans-membrane domain and a 206 amino acid cytoplasmic domain. The cytoplasmic regions of both TRAIL-R1/DR4 and TRAI-R2/DR5 consist of a death domain (DD) homologous to Fas and TNF-R1, and DR4 and DR5 can transduce apoptotic signalling cascades after binding to TRAIL. However TRAIL-R3/DcR1 and TRAIL-R4/DcR2 lack the intracellular death domain, therefore they don't have the capability of inducing apoptosis by binding to TRAIL, even though they contain the extracellular domains which are highly homologous to those of DR4 and DR5. Recent studies have indicated that TRAIL-induced apoptotic signal pathway is similar to CD95-mediated apoptosis (Wang, 2003; Kuang, 2000; Sprick, 2000; Shankar, 2004).

1.2.2 DISC (death-inducing signalling complex)

1.2.2.1 DISC formation in CD95- mediated apoptotic signal pathway

CD95 is type I transmembrane molecule and has three cysteine-rich extracellular domains and an intracellular death domain (DD), the DD was found in all death receptors, which is essential for signalling (Smith, 1994). CD95 contains an approximately 80 amino acids death domain (DD).

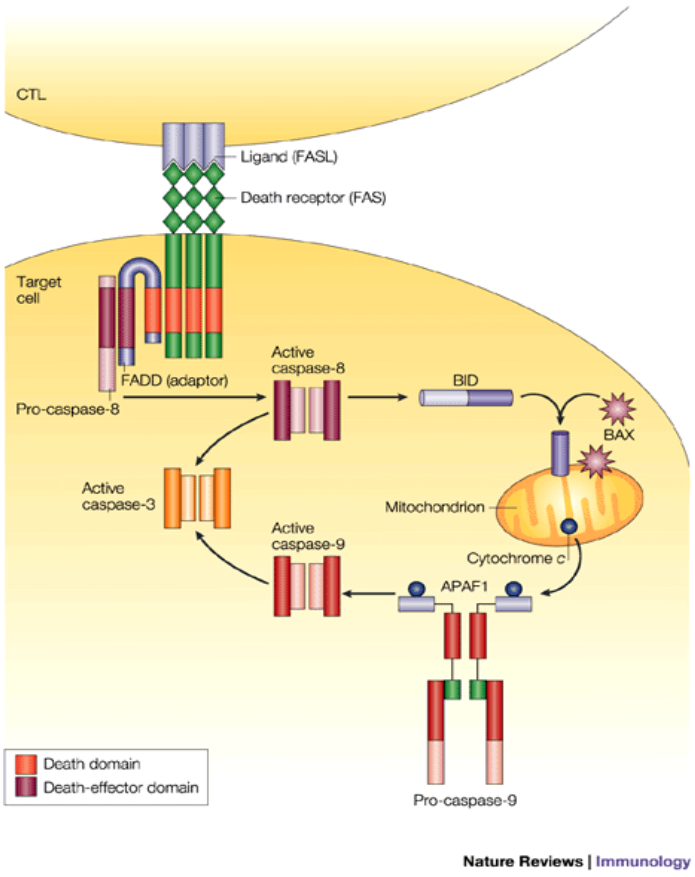


Figure 1: The CD95/Fas mediated apoptotic signalling pathway

Figure 1 shows the CD95-mediated classical apoptosis signalling pathway. The CD95-mediated apoptosis signal pathway is activated through CD95 receptor cross-linking either through specific ligand (CD95L) or with agonistic antibody (anti-APO-1), which will trigger downstream signal components and lead the cell into death. The structure formed from ligand or agonistic antibody, trimerized CD95-receptor and other associated signal molecules is termed the death-inducing signalling complex (DISC) (Kischkel, 1995). For the beginning of the signal cascade, the adaptor molecule FADD (Fas associated death domain) and the initiator caspase, procaspase-8 are first recruited. In addition to the DD (death domain), the adaptor molecule FADD possesses an amino-terminal, and it is called DED (death effector's domain), which is essential for the transduction of apoptosis signal. The procaspase-8 possesses two DEDs.

Through homologous interaction of the DEDs, the procaspase-8 was cleaved at the DISC and lead to the formation of active caspase -8.

1.2.2.2 DISC formation in TRAIL- mediated apoptotic signal pathway

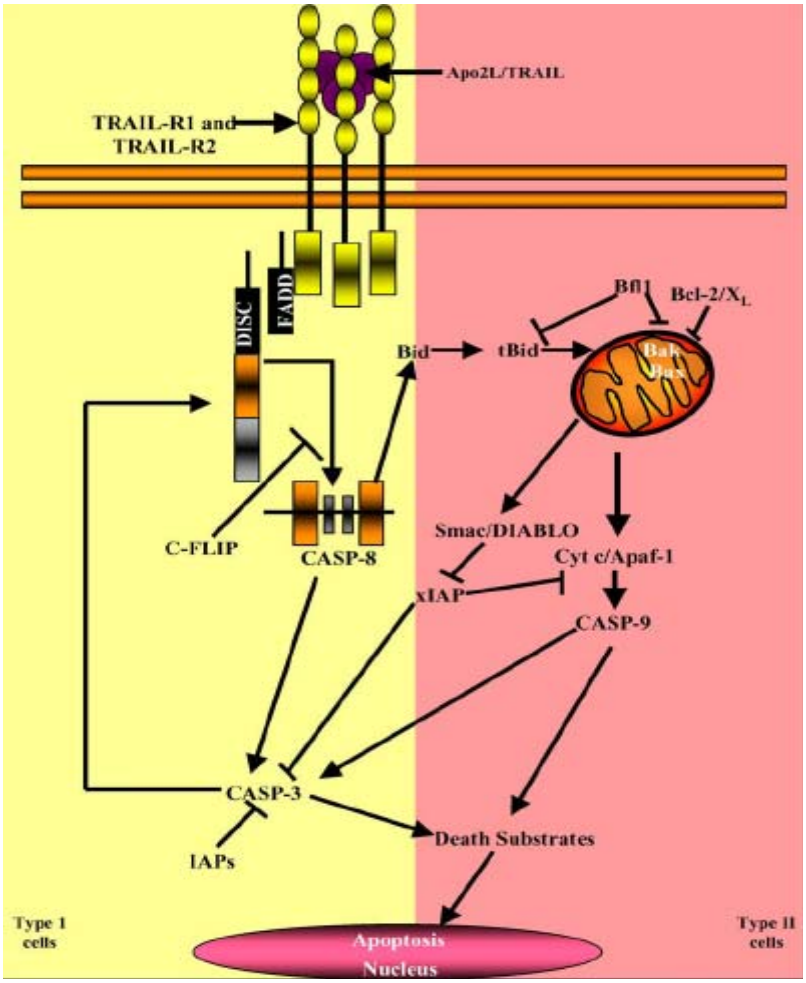


Figure 2: The signalling pathway of TRAIL-induced apoptosis in the cells

(Figure from Bouralexis, 2004)

The Figure 2 shows the classical signal pathway triggered by TRAIL. Through binding to the TRAIL will induce trimerization of DR5 or DR4 on the surface of the responsible target cells, which give rise to the formation of death-inducing signalling complex (DISC). Fas-associated death domain (FADD) is also required for TRAIL-induced apoptosis signal (Kuang, 2000; Kischkel, 2000). The trimerization of the death

domains lead to recruitment of the adaptor FADD, which in turn recruits and activates pro-caspase-8 into an active form. In type I cells, the activation of caspase-8 is sufficient for subsequent activation of the apoptotic effector caspase-3, which executer cellular apoptosis signal. In type II cells, this signalling pathway is involved with cleavage of Bid by caspase-8 and translocation of Bid into mitochondria, cytochrome c release from mitochondria. This released cytochrome c binds to Apaf-1 to activate caspase-9, which can activate the apoptotic effector caspase-3, finally the cells is induced to death.

There are some factors or proteins involved in regulating TRAIL-mediated apoptosis. It has been recently reported that nuclear factor *k*B (NF- *k*B) can regulate the sensitivity of target cells to TRAIL-induced apoptosis in hepatoma cells (Shigero, 2003) through DR5 up-regulation and NF- *k*B inhibition by interferon-α. Some clinical experiments showed that proapoptotic protein Bax and anti-apoptotic proteins Bcl-2 and Bcl-X_L were also associated with regulating the TRAIL-induced apoptosis signal. As an important messenger, nitric oxide (NO) can sensitize carcinoma cell lines to TRAIL-mediated apoptosis via inactivation of NF- *k*B and inhibition of Bcl-xL expression (Huerta-Yepez, 2004) or through mitochondria-dependent pathway in human colorectal carcinoma CX-1 cells (Lee, 2001). Furthermore, chemotherapeutic drugs and radio-therapeutic method can also sensitive tumor cells to TRAIL.

Because TRAIL shows anti-tumor activity with little cytotoxicity to normal tissues or cells, the soluble recombinant TRAIL (rTRAIL) has been used as a cancer therapeutic tool. But some cancer cell lines and most primary tumors are resistant to rTRAIL-induced apoptosis, and several backgrounds for this resistance have been reported, the lack of the expression of DR4 and DR5 may be the central role (Shankar, 2004).

1.2.3 Caspases

Caspases are the main molecular executors of apoptosis. In humans there are together 13 different enzymes, which are synthesized as pro-enzyme form and activated through proteolytic cleavage. Using a peptide library for specificity analysis, caspases are

classified into three groups that may have redundant functions: first group, caspase-1, -4 and -5; second group, caspase-3, -7 and -2; third group, caspase-6, -8 and -9.

In both CD95- and TRAIL-induced apoptosis, caspase-8 plays a central role. It was directly activated at the DISC. Procaspase-8 activation generates active caspase-8 by catalytic cleavage, and caspase-8 has different effects in different cells of the Type I / II system. In type I cells, it was able to activate other members of the caspase family, such as caspase-3, directly (Scaffidi, 1998). But in type II cells, caspase-8 triggered cleavage of the proapoptotic Bcl-2 family member Bid, and the translocation of Bid to the mitochondria. After Bid located into the mitochondria membrane, it will induce the release of mitochondria cytochrome c, which lead to the activation of caspase -9 through interaction with the adaptor molecule apoptotic protease-activating factor 1 (APAF-1), then caspase-9 activate caspase-3. Caspase-3 may activate caspase-6. Both caspase-6 and -3 cleaved proteins involved in shrinkage and fragmentation of apoptotic nuclei (Hirata, 1998), finally it triggered the cells to apoptosis.

1.2.4 Mitochondria

Mitochondria, the energy-producing organelle in the cells, is tightly involved in the process of apoptosis, and many events will lead to apoptosis which are associated with mitochondria, such as the release of cytochrome c, disruption of electron transport and energy metabolism, loss or changing of mitochondrial transmembrane potential, alteration of cellular oxidation-reduction (redox) state (Lee, 2000). The initiation of mitochondria-involved apoptosis can be prevented by anti-apoptotic molecules such as Bcl-2 and Bcl-X_L or promoted by molecules containing Bax and Bid, 2 pro-apoptotic proteins of Bcl-2 family, which is related with mitochondria-associated apoptosis signal.

In both CD95- and TRAIL-mediated apoptotic pathways, the signal cascades involved with the mitochondria is nearly same. When the cell was triggered into initiation of apoptosis, caspase-8 was firstly activated. Cytosolic Bid translocated into mitochondria and interacted with mitochondrial membrane after activation by caspase-8 (Green, 1998; Tafani, 2002). The change of membrane permeability lead to the reduction in the

mitochondria membrane potential ($\Delta\Psi_m$) and mitochondrial proteins were then released. Cytochrome c, one of the released proteins, associated with Apaf-1 and procaspase-9 may lead to form active caspase-9 through autocatalytic activation and this process is ATP-dependent (Hengartner, 1998). On the contrary, Bcl-2 and Bcl-XL may hinder this release of cytochrome c and prevent cells from apoptosis (Sun, 2002; Poulaki, 2001; Tafani, 2002).

Bcl-2 and Bcl-X_L may hinder the release of cytochrome c and prevent cells from apoptosis (Sun, 2002). Furthermore, from the experiment of Bid-deficient mouse (knock-out mouse) showed that these $Bid^{-/-}$ cells were resistant to CD95-induced hepatocellular apoptosis (Yin, 1999; Adrain, 2001), and others reported that Bcl-2 inhibited Bid-induced apoptosis at the mitochondrial level by blocking cytochrome c release and reduced CD95-induced apoptosis sensitivity. Those results supported the important role of mitochondria in apoptosis signal pathway (Hawkins, 1994).

1.2.5 Endoplasmic reticulum

The endoplasmic reticulum (ER) is mainly responsible for folding and modifing proteins for secretion to the cell-surface. Recently, many studies demonstrated that endoplasmic reticulum (ER) stress is related with some human diseases, such as Alzheimer's disease (AD), Parkinson's diseases, and cystic fibrosis and neuronal damage by ischema (Katayama, 1999; Wigley, 1999; Imai, 2000, 2001; Nakagawa, 2000; Sato, 2001; Tamatani, 2001). ER stress may be induced by various factors, such as depletion of calcium ($Ca2^+$) from the ER lumen, expression of mutant proteins or accumulation of misfolded proteins and ischemia (Imaizumi, 2001). To survive ER stress, there are three major cellular responses: ER-related degradation, unfolded protein response (UPR) and apoptosis (under high ER-stress conditions). Under ER-stress, the cells may up-regulate the genes which are responsible for reducing overall translation or regulating the protein folding process (Kaufman, 2002; Forman, 2003), or the cells can reduce stress through enhancing the proteasomal degradation of misfolded ER proteins in cytosol and decreasing the accumulation of misfolded proteins, finally relieving cells from stress (Bonifacino, 1998; Travers, 2000; Kozutsumi, 1998). Furthermore, under long-term or high ER-stress, the cells may be

induced to undergo apoptosis (Imaizumi, 2001) through ER-stress specific caspase-4 activity (Hitomi, 2004).

There are several ER stress-associated proteins or factors, such as GADD153/CHOP (growth arrest and DNA damage factor / CAAT/enhancer binding protein homologous transcription factor), BiP, VCP and caspase-4, involve in the regulation of ER stress (Zinszner, 1998). Valosin-containing protein (VCP) and apoptosis-linked gene-2 (ALG-2) were reported to play an important role in mediating ER stress-induced cell death (Rao, 2003). In mouse, caspase-12 is described in response to ER stress (Nakagawa, 2000), although human caspase-12 gene is transcribed into mRNA, the mature and functional caspase-12 protein is not produced, because the gene is interrupted by a frame shift and premature stop codon (Fischer, 2002), and thus in human, caspase-12 is not related with ER stress-induced apoptosis. Human caspase-4 was cloned and recognized as the most homologous caspase to mouse caspase-12, both molecules belong to the caspase -1/ICE subfamily within the caspase family (Kamens, 1995; Lin, 2000). Human caspase-4 is mainly located in ER membrane and involved in ER stress-induced apoptosis in human (Hitomi, 2004). Transcription factor GADD153/CHOP is lower expressed under favorable conditions, and ER stress as a result to UPR, increases the transcription of GADD153/CHOP, which is closely associated with apoptosis (Zinszner, 1998).

Overexpression of calreticulin, an ER luminal protein, can sensitize the cells to apoptosis induced by thapsigargin (a reagent that promotes ER stress through depletion of lunimal calcium stores). This correlated with an increased release of cytochrome c from mitochondria. calreticulin deficient cells were significantly resistant to apoptosis, correlating to a decreased release of cytochrome c from mitochondria and low levels of caspase -3 activities (Nakamura, 2000).

1.3 Nitric oxide (NO)

Nitric oxide has important roles in human nerves, vascular system and immune response in physiological and pathological situation. NO synthesis is part of the inflammatory response against pathogens, such as bacteria, viruses and tumor cells. As

a free radical molecule, after massive NO formation, NO function as a messenger, regulatory, cytostatic and cytotoxic molecule, it affect signalling pathways, induces cell death or has a protecting role during apoptotic cell death (Moncada, 1991; Lowenstein, 1992; Murphy, 1999).

1.3.1 The biosynthesis of nitric oxide (NO)

There are three different nitric oxide synthases (NOS), two of them are constitutively expressed in characteristic cell types, and these NO synthases are termed nNOS and eNOS respectively. nNOS are predominantly expressed in neurons and also called NOS1, eNOS are mainly expressed in endothelial tissue and also termed NOS3. The last nitric oxide synthase isoform is an inducible form (iNOS), it is also termed NOS2. It can be induced in immune cells or other tissues under a cellular immune response. All of these nitric oxide synthase isoforms demand L-arginine as a reaction substrate and need other co-factors, including NADPH, tetrahydro-biopterin (BH_4), FAD and FMN (Gross, 1995; Kroncke, 1997; Mayer, 1997; Knowles, 1994).

The level of intracellular calcium seems to be important in the enzymatic activation of nitric oxide synthases. In that, the expression of nNOS (NOS1) and eNOS (NOS3) are significantly regulated by the level of intracellular calcium. The inducible NO synthase (iNOS/NOS2) seems to be fully activated even at the low level of Ca^{2+} in cells, this appears that NOS2 is less sensitive to the level of calcium. However other factors were recognized that also modulated the activity of those enzymes, such as phosphorylation, palmitoylation and intracellular localization of nitric oxide synthase enzymes (Bredt, 1990; Yamamoto, 2004; Nathan, 1992; Förstermann, 1991; Evans, 1992). NOS2 was regulated also at the level of gene expression, NOS2 promoter activity and NOS2 mRNA stability were important for this regulation of NOS2 expression (Kleinert, 2003; Förstermann, 2003).

Nitric oxide synthase produces not only the major product, NO, but also other molecules coming from the oxidation , reduction or adduction of NO in physiological settings, therefore it produces diverse nitrogen oxides, S-nitrosothiols, peroxynitrite ($ONOO^-$), and transition metal adducts. For NO-signalling pathway analysis, NO

synthase inhibitors such as N^G-monomethyl-L-arginine (NMMA) are used to interfere with NO production (Beckman, 1996; Hausladen, 1994; Jia, 1996; Stamler, 1994).

For investigating signalling pathways, NO-releasing drugs or compounds are used as study tools for this purpose. Nitric oxide (NO) releasing compounds are normally termed NO donors, which produce NO and trigger the biological activity after their decomposition. The common NO donors, such as 3-morpholinosydnonimine (SIN-1), S-nitrosothiols, sodium nitroprusside and S-nitroso-N-acetylpenicillamine (SNAP), are generally used to produce NO in experiments.

As small and hydrophobic free radical, nitric oxide (NO) can pass through membranes and diffuse both within and between cells. The transcellular diffusion of NO is much faster than its intracellular reaction, and this provides the opportunity to react with other target molecules (Dawson, 1996; Lancaster, 1997; Beckman, 1996).

1.3.2 Nitric oxide (NO) and apoptosis

Nitric oxide (NO), produced from activated macrophages, is a key and main cytotoxic molecule in the defense against pathogens, viruses, bacteria, fungi and tumor cells. In macrophages, NO production is mainly dependent on L-arginine. The more detailed mechanisms about cell apoptosis caused by nitric oxide (NO) are still not clear. Many studies showed that NO-mediated apoptosis was more complicated process and it was involved multiple pathways, the apoptosis sensitivity to NO changes significantly from one cell type to another. In a typical apoptotic alteration, the signalling components which are involved with NO are the accumulation of the tumor suppressor protein p53, DNA damage, morphological change, loss of mitochondrial membrane potential, and may influence ER stress (Oren, 1997; Wang, 1997; Meßmer, 1994; Meßmer, 1996; Ho , 1996; Zhao, 1997; Rao, 2004;).

In former studies, it was shown that NO targets naked DNA and induces oxidative DNA damage in activated macrophages; NO-induced cellular DNA damage will trigger a stress response in mammalian cells (Nguyen, 1992; Wink, 1991). Poly (ADP-ribose) polymerase (PARP) is involved in NO caused DNA damage. Damaged-DNA

activated PARP, and also up-regulated p53. PARP activation is a key step in NO cytotoxicity in the cells. Massive PARP stimulation result to the ADP-ribose donor, NAD^+, depletion. The following NAD^+ resynthesis make the ATP depletion, finally the main consequence of DNA damage by nitric oxide (NO) is energy deprivation in the cells (Althaus, 1987; DeMurcia, 1994; Henry, 1993). The tumor suppressor gene p53 is known as one of the important guardians of the genome and a member of the DNA damage-response pathway. P53 can induce growth arrest or apoptosis in DNA damaged cells. Induction of G1 arrest dependents on specific DNA binding and transcriptional activation of p53 target genes such as p21 (Oren, 1997; White, 1994; Almog, 1997). p21 is an inhibitor of cyclin dependent kinases, and therefore p21 can blocks the cell cycle process (Liebermann, 1995; Shankland, 1997). In DNA damage caused by nitric oxide (NO), the tumor suppressor protein p53 also accumulates. For example in RAW 264.7 macrophages NO produced endogenously or from NO donors leads to p53 accumulation during DNA damage process. P53 accumulation causes growth arrest by blocking the G1/S phase transition in the cell cycle, and this arrest gives time to the cells for DNA repair. However, if DNA damage caused by NO is significantly extreme, p53 will induce the cell to undergo apoptosis (Meßmer, 1996b; Meßmer, 1994).

Mitochondria are energy producing organelles and are composed of two membranes, a matrix and its own DNA (mtDNA).The main function of mitochondria is to supply the energy in form of ATP for the cell through the respiratory chain in the inner membrane by using oxygen. The cell will undergo apoptosis or necrosis after mitochondrial dysfunction induced from a variety of stimuli. As a free radical molecule, nitric oxide (NO) can influence on mitochondrial functions (Packer, 1997; Bates, 1995). Reactive peroxynitrite generated by the reaction of nitric oxide with superoxide was related with NO-induced neuronal cell apoptosis, and also other studies showed the dysfunction of mitochondria involving nitric oxide caused cells to undergo apoptosis such as PC12 cells, macrophage, and several other systems. Nitric oxide (NO) caused apoptosis or cell death through mitochondrial by the following processes: 1) NO changed or decreased the mitochondrial membrane potential $\Delta\psi_m$ (Takabayashi 2003; Solenski, 2003). This depolarization of mitochondrial membrane potential loss induced by nitric

oxide (NO) can be observed on the FACS analysis by staining with JC-1 dye. 2) Cytochrome c release from mitochondria and formation of a complex of cytochrome c, apoptotic protease activity factor-1 (Apapf-1) expression and caspase -9 activations, which triggers the cleavage and activity of caspase -3 and leads to apoptosis (Kroemer, 1997; Kluck, 1997; Yang, 1997; Hsu, 1997; Jurgensmeier, 1998). 3) Influence on the mitochondrial electron transfer and ATP synthesis through inhibiting cytochrome oxidase, causes alterations in mitochondrial complexes which limits energy output, causeing to apoptosis (Hausladen, 1994; Lizasoain, 1996; Cassina, 1996; Radi, 1994).

Some other factors were also related with cytochrome c release from mitochondria, such as proapoptotic proteins Bax and Bak. When those proteins interacted with the outer membrane of mitochondria could lead to the release of cytochrome c. In some studies showed that nitric oxide could enhance Bax or Bak proteins expression (Meßmer, 1996c), and through this indirectly influence, nitric oxide caused cytochrome c release and finally triggered downstream signalling activations.

Recently a number of studies have focused on the relationship between endoplasmic reticulum stress and cell apoptosis (Oyadomari, 2002). As a crucial organelle of protein synthesis and transportation in the cells, endoplasmic reticulum (ER) stress from a wide variety of stimuli may influence the normal function of cells. Nitric oxide induced apoptosis is generally considered to be involved with DNA damage or mitochondria pathway, but other studies have shown that endoplasmic reticulum stress pathway is also involved in NO-mediated apoptosis. The apoptosis associated with ER stress can occur through three ways. The first way is related with the transcriptional factor GADD153/CHOP (C/EBP homologous protein), a member of the C/EBP family of transcription factors, and the Ca^{2+} level in the ER lumen. CHOP is expressed at low level under normal physiological condition, but it is strongly induced in response to ER stress, and overexpression of CHOP will lead to growth arrest and maybe to apoptosis (Ron, 1992; Wang, 1996; Barone, 1994; Matsumoto, 1996). The second way involved with ER stress is activation of the cJUN NH_2-terminal kinase (JNK). Nishitoh reported that ASK1 (apoptosis signal-regulating kinase) is essential for endoplasmic reticulum stress-induced neuronal cell death (Nishitoh, 2002). Sustained activation of the JNK and p38 pathway requires the activation of ASK1, and which leads to apoptosis

(Tobiume, 2001). Another ER stress-related pathway involves caspase-12 (in mice) and caspase-4 (in humans). Many studies showed that the activation of caspase-12 is associated with ER stress-induced apoptosis in mice (Yoneda, 2001; Nakagawa, 2000; Bitko, 2001). Hitomi reported that human caspase -4 is an ER stress-specific caspase in human cells (Hitomi, 2004), and the activation of caspase -4 will induce apoptosis.

Besides these three main apoptotic signalling way involved in ER stress-associated apoptosis, other factors or proteins were also reported in response to NO-mediated ER stress such as BiP, Bak and Bax. Studies have revealed that Bax and Bak can localize to the endoplasmic reticulum to initiate apoptosis (Zong, 2003). Some experiments showed that the transcription factor GADD153/CHOP, and ATF6, exists constitutively as the transmembrane protein p90ATF6 in ER under non-stressed condition, are involved in NO-induced apoptosis (Gotoh, 2002; Oyadomari, 2001). Other studies also showed that ER calcium stores are a primary target of NO toxicity in the cells, intracellular Ca^{2+} pool depletion is linked to the induction of NO synthesis in murine peritoneal macrophages (Doutheil, 2000; Park, 1995). Endogenously generated NO, which disrupts the respiratory chain, may cause changes in mitochondrial calcium flux, these changes will induce the cleavage of the endoplasmic reticulum (ER) stress-regulated transcription factor p90 ATF6. This indicates that NO-dependent mitochondrial disruption is coupled to the ER stress response (Xu, 2004). However the exactly mechanism involving NO in ER stress-induced apoptosis is still not clear.

1.3.3 NO and immune surveillance

The expression of nitric oxide has been found in different tissues or cells, which were involved in the immune response, such as macrophages, dendritic cells, natural killer (NK) cells and tumor cells of B or T cell origin. Nitric oxide has been termed as one of the most important factors in the immune response. It is involved in the pathogenesis, against bacteria infection, inhibition of tumors and it is also related with autoimmune processes and chronic degenerative diseases (Bogdan, 2001).

In the maturation of T cells in the thymus, NO may play a crucial role as effector molecule, because of the ability to trigger apoptosis in the cells, NO might be involved in the selection and development of T cells in the thymus. Some studies indicates that

TCR-activated double-positive thymocytes are very sensitive to NO-induced apoptosis, therefore, NO produced from NOS2 expression is one of the important factos regulating deletion of double-positive thymocytes (Tai, 1997; Moulian, 2001; Fehsel, 1999).

Induction of apoptosis in tumor cells or inhibition of tumor growth by activated macrophages is one of the crucial NO functions of the immune system. Tumor cells can be induced to undergo apoptosis by iNOS expression within tumor cells in response to INF-γ and TNF, which were released by cytotoxic T lymphocytes (Homma, 2005; Immunology, Fifth Edition). NO from transfection of NOS2 gene prevents tumor metastasis and induces regression of established tumors in vivo in certain melanoma or sarcoma cells. However, the expression of NOS2 was also frequently found in various tumor cells, where it enhances tumor growth, invasiveness and may work against an anti-tumor immune response. Besides induction of apoptosis in tumors, NO shows the functions of antiviral, antimicrobial, cytotoxic and cytopreotective effects in the infected host organisms. During inflammation NO produced from the inducible NO synthetase (NOS2/iNOS) in response to pro-inflammatory cytokines is critical for short term defense against infection and tumor cells. The expression of iNOS is found in a broad number of inflammatory diseases or tumors in humans, such as breast cancer, melanoma, bladder cancer, hepatocellular carcinoma, and colorectal cancer which often express high levels of NOS2 (Ekmekcioglu, 2000; Vakkala, 2000; Wolf, 2000; Yagihashi, 2000). The underlying roles that NO plays in tumor-host cell interactions are complex and may be dependent on the context and source of NO. For example, recent data using a genetic strategy in mice has provided evidence that NO can suppress tumorgenesis as p53-/-NOS2-/- mice developed tumors more rapidly as the corresponding wild type mice (Hussain, 2004). Furthermore, anti-tumor activity through NO production has been attributed to host macrophages. On the other hand, significant experimental and clinical evidence suggest that tumor-derived NO is conducive to tumor progression and detrimental to the host (Albina, 1998; Edwards, 1996; Moochhala, 1999; Cianchi, 2003; Wenzel, 2003).

1.4 The goal of this study

NO can trigger apoptosis in many in vivo and in vitro experimental models (Kim, 2001; Hussain, 2003). But the apoptotic signalling pathway involved with NO in CD95- or TRAIL-mediated apoptosis are still not completely understood. Some studies showed that nitric oxide can increase the apoptosis sensitivity in CD95- or TRAIL-induced signalling pathway, while other experiments indicated that NO functioned as a anti-apoptotic factor this apoptosis signal.

The effects of endogenous nitric oxide (NO) in the apoptotic signalling pathway, especially in the CD95- and TRAIL-mediated apoptosis are investigated and the hypothesis that chronic exposure to NO induces apoptosis and selects for cells with reduced sensitivity to NO and apoptosis was tested. The human NOS2 cDNA was cloned into the ecdysone-inducible plasmid and transfected into EcR293 cells to establish a stable cell lines. Cell lines was selected that displayed ecdysone-inducible expression of the NOS2 gene. With these cells lines to study the influence of NO on CD95- or TRAIL-mediated apoptosis sensitivity, cytotoxicity, cell growth and the action of cytotoxic T cells during short term exposure to NO was investigated. Cells under chronic exposure to NO were selected; the apoptosis sensitivity and CTL killing were analysed.

2 Material and Methods

2.1 Instruments and equipments

	Name	Company or From
1	Bosch -20 ^{o}C refrigerator	Germany
2	GFL type 1002 waterbath	Germany
3	Megafuge 1.0R	Laborgeräte Vetter GmbH, Germany
4	Binder cell culture incubator	Tuttlingen, Germany
5	Electrophoresis apparatus (agarose gel)	OWL scientific for Labotec, Wiesbaden, Germany
6	Centrifuge apparatuses for Eppi	Hamburg, Germany
7	Flurometer spectraFluorPlus	Tecan, Crailsheim
8	pH-Meter Toledo 320	Mettler Toledo, Gießen, Germany
9	Cell culture microscope wilovert 30 standard	Hund, Wetzlar, Germany
10	Cell culture cabinet	Allerod, Denmark
11	GFL type 1004 waterbath	Germany
12	Eppendorf centrifuge 5840	Eppendorf, Germany
13	-80 ^{o}C refrigerator	Ultra-Low technology of laboratory equipment, USA
14	Electrophoresis power supply EPS 601	Amersham pharmacia biotech, Sweden
15	Cell culture flowbank lamin air (type S-2000 1.5)	Heto-Holten, Danmark
16	UV/Visible spectrophotometer ultrospec 3000	Pharmacia biotech, Germany
17	Digital camera system cybertech CSI	Hitachi, Japan
18	Microflow cell cabinet	Germany

19	Electrophoresis power supply	Micro Bio Tec Brand, Gießen, Germany
20	Leiva light microscope	Leica, Portugal
21	Turning-fork scale	Japan
22	Heidolph unimax 1010	Germany
23	Innova 4230 refrigerator incubator shaker	New Brunswick Scientific, USA
24	Centrifuge 5405 R for small Eppi	Eppendorf, Germany
25	Folienschweissgerät Vacupack plus	Krups, Solingen, Germany
26	Confocal laser scan LSM 510-UV microscope	Zeiss, Oberkochen,Germany
27	UV-Transilluminator	Renner, Damstadt, Germany
28	PCR machine	Oldendorf, Germany
29	Shaker	IKA-Labortechnik, Germany
30	Film developer machine	Fujitsu, Japan

2.2 Chemicals, solutions and materials

	Name	**Company**
1	Protein concentration determination kit	Pierce, Germany
2	Griess assay reagent	Alexis Biochemicals, USA
3	CellTiter-Glo luminesence cell viability assay kit	Promega, USA
4	DMEM (Eulbecco's modified Eagle's medium)	Invitrogen, UK
5	FCS (fetal calf serum)	Invitrogen, UK
6	Ponasterone A	Invitrogen Corporation, USA
7	Lipofectamine 2000 reagent	Life technology, USA
8	Tris	Carl Roth GmbH, Germany

9	Bacto Agar, Yeast Extract, Bacto Trypotone	Becton Dickinson, USA
10	Sodium nitrite	Sigma chemical Corp., USA
11	Geneticin (G418)	Invitrogen Corp, UK
12	Ammonium persulfate	Sigma chemical Corp. USA
13	Zeocin	Invitrogen , USA
14	Various enzymes	Sigma, Promega
15	1x PBS for cell culture	Cambrex, Belgium
16	Hepes buffer for cell culture	Cambrex, Belgium
17	L-Glutamine for cell culture	PAA, Pasching, Austria
18	Trypsine/EDTA	PAA, Pasching, Austria
19	Penicillin/Streptomycin	PAA, Pasching, Austria
19	Protein A	Sigma, Germany
20	1X PBS –EDTA for cell culture	Cambrek, Belgium
21	1% NP-40 for lysis of cells (1% NP-40 in 1X PBS, added 1x protein inhibitor)	Sigma, Steinheim, Germany
22	CellTiter-Glo assay kit	Promega, USA

2.3 Antibodies

2.3.1 Primary antibodies for immunostaining and Western blot

	Name	**Company or Come from**
1	rabbit anti-NOS2 polyclone antibody	Santa Cruz Biotechnology, USA
2	mouse anti-caspase 3 monoclone antibody	Apotech Corporation, Germany
3	mouse anti-caspase 8 monoclone antibody	Apotech Corporation, Germany
4	mouse anti-caspase 9 monoclone antibody	Apotech Corporation, Germany
5	anti-BiP polyclonal antibody	Affinity Bioreagents Inc, USA

6	α-Actin antibody	Biocarta, USA
7	anti-Apo-1	DKFZ, Germany (Prof. Krammer)
8	anti-caspase-4	Santa Cruz Biotechnology
9	anti-CHOP/GADD153	Santa Cruz Biotechnology
10	anti-VCP monoclonal antibody	BD, USA
11	ALG-2 polyclonal antibody	Swant, Switzerland
12	anti-TRAIL-1 (for receptor staining)	Alexis, USA
13	anti-TRAIL-2 (for receptor staining)	Alexis,USA

2.3.2 Immunoglobulin secondary antibodies for Western blot

	Name	company or From
1	anti-rabbit immunoglobulin alkaline phosphotase-conjugated secondary antibody	Sigma-Aldrich Corp, USA
2	anti-mouse immunoglobulin alkaline phosphotase-conjugated secondary antibody	Sigma-Aldrich Corp, USA
3	anti-goat immunoglobulin alkaline phosphotase-conjugated secondary antibody	Sigma-Aldrich Corp, USA

2.3.3 Fluorescence secondary antibodies and fluorescence staining dye

	Name	Affinity to	Company or From
1	Hoechst 33342	Nucleus	Molecular Probes, Leiden, NL
2	Alexa 488 goat-anti-mouse IgG	Mouse IgG	Molecular Probes, Leiden, NL

2.4 Software

Microsoft Office 2000	Macromedia Freehand 8.0.1	Zeiss LSM 510
Adobe Photoshop 5.0	BD CellQuest Pro	Tecan Xfluor
MagicScan V 4.5	Endnote 7.0	Adobe Acrobat 6.0

2.5 Molecularbiological and biochemical methods

2.5.1 Cloning of target gene

2.5.1.1 Enzymes and plasmids

Restriction endonucleases: Roche, Mannheim; Biolabs, Frankfurt

T4-Ligase	Roche, Mannheim
CIAP	Promega, Mannheim
DNA-polymerase	Promega, Mannheim
Oligonucleotide	MVG, Ebersberg

- pTRE-NOS2-cds

From Dr. Hartmut Kleinert, Uniklinik Mainz

- pBluescript II KS+/- (Invitrogen,Germany)

- Cloning vector and ampicillin resistance in bacteria

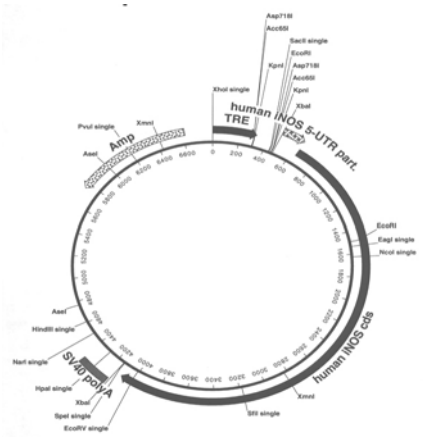
- pIND (SP1)

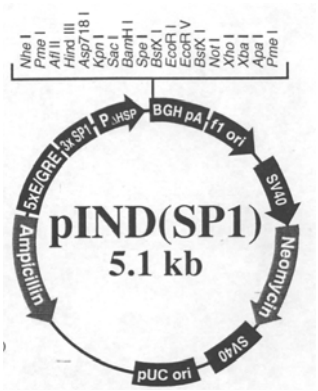
- Invitrogen, Karlsruhe, Germany

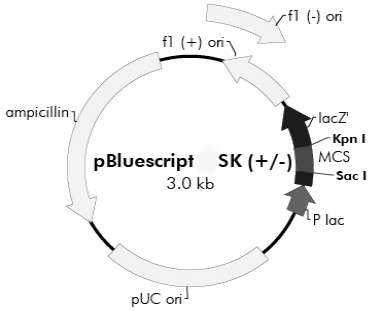
- Expression vector for mammalian cells, and allowed the inducible gene expression in HEK293 cell lines

- G418 resistance for stable expression in mammalian cells

- Ampicillin resistance in bacteria







2.5.1.2 Isolation of plasmid DNA:

According to the protocol from Qiagen company, 2 ml LB medium containing E.coli was cultured overnight for obtaining small amount plasmid DNA, and the isolation was performed with QIAprep Miniprep Kit (Qiagen, Hilden, Germany). For obtaining large-scale plasmid DNA, 25 ml E.coli LB medium was incubated overnight and the plasmid DNA was isolated with Qiagen Plasmid Midi Kit followed by the manual.

The DNA isolation is based on alkaline lysis of bacteria, and DNA was absorbed and purified through the silicium-matrix cylinder, the isolation process was followed according to the manual of Qiagen company.

2.5.1.3 Phenol-chloroform extraction

The DNA solution was mixed with an equal volume of phenol/chloroform (1:1) (adjusted with 10 mM Tris to pH 8.0). The solution was vortexed for short time until the solution become viscous. Afterwards the mixture was centrifuged at 5000 g for 1 min. There were two phases. The upper phase was carefully transferred into a new tube, and the lower phase was discarded.

The above purification steps were repeated once. Finally an equal volume of chloroform was added the supernatant to remove the rest of phenol. After vortexing the mixture, the solution was centrifuged one time at 5000 g for 1 min, and the supernatant containing purified DNA was carefully transferred into a new tube.

2.5.1.3 DNA precipitation

The DNA solution was added with 0.5 volume 7.5 M ammonium acetate pH 7.5 and addition with 2.5 volumes 100% ethanol. Then the solution was stored at -20 °C for 2 hours. Afterwards it was centrifuged at 4 °C at 12000 g for 20 min, and DNA pellet was washed one time by ice-cold 70% ethanol, dried, and dissolved in a proper volume of pure water, and the solution containing DNA was stored at -20 °C for analysis or future experiments.

2.5.2 Agarose gel electrophoresis

DNA marker MBI Fermentas, St.Leon-Rot
Gene Ruler 1 kb DNA ladder

TBE (Tris-Borat-EDTA-buffer)

Tris-HCL pH 8.0 .. 90 mM
Boric acid 90 mM
EDTA pH 8.0 .. 2.5 mM

Loading buffer for DNA agarose gel

0.2% Brompheoolblue
30% Glycerol
100 mM EDTA, pH 8.0

The digestion of DNA was performed with 5-10 units endonuclease per µg of DNA in the recommended reaction buffer and temperature for 1-2 h according to the instruction from the company.

The electrophoretic separation of DNA fragments were performed in agarose gel from 0.8% to 2%, and with addition of 0.1 µg/ml ethidium bromide (from a stock solution of 10 mg/ml in water) to mix thoroughly. Before electrophoresis, DNA was mixed with 1/6 volume DNA loading buffer. The electrophoresis buffer was 1 X TBE buffer, and molecular weight marker was used for checking the size of the DNA fragments. The electrophoretic results were documented with digital camera after observation under the UV-Transilluminator.

2.5.3 Isolation fragments from agarose gel

The target DNA fragments and vector were first digested by appropriate restriction enzymes. After digestion the DNA fragments were separated on an agarose gel, and the target bands were cut with a sharp knife. The DNA from the cleaved agarose gel was isolated with GenElute Gel Purification Kit (Sigma, Taufkirchen, Germany) according to the instructions from the company. Instead of gel isolation, the vector was purified by phenol-chloroform extraction and precipitated by ethanol.

2.5.4 Subcloning of DNA fragments

For cloning the vector was cleaved by the appropriate restriction enzyme, and dephosphorylated by calf intestinal alkaline phosphatase (CIAP) to remove 5'-phosphate groups from linear DNA, and this process prevents the recircularization and religation by itself. DNA was resuspended in purified H_2O; afterwards CIAP and the appropriate buffer were added, incubated under the appropriate conditions. For the inactivation of the CIAP enzyme, EDTA (pH 8.0) and SDS were added to a final concentration of 20 mM and 0.5% respectively after dephosphorylation, and the solution was incubated at 70 ^{o}C for 10 min. For purification of the vector, the phenol-chloroform method was performed, and precipitated by ethanol. Afterwards the ligation of the vector and target insert fragment was completed as following:

The ratio of insert fragment and vector is from 3:1 to 10:1. The maximum amount of the vector is 100 ng and the amount of insert fragment was variable. The responsible amount of T4-DNA-ligase (1 U/µl) and 10 X ligase-buffer is 1 µl respectively. The reaction of ligation was started in a final volume of 10 µl.

2.5.5 Preparation and conservation of competent bacteria

LB-Medium (1L)

-Trypton 10 g
-Yeast 5 g
-Natriumchlorid 10 g
-Add distilled water until to 1 L
-and adjust pH 7.0 with NaOH, then the LB-medium was autoclaved.

LB-Agar plates

-LB-Medium + 15 g Agar per 1 liter volume
-Autoclaved
-and the antibiotic was added after the solution cooling down to 50-60 ^{o}C

Antibiotics

The stock solution was stored at - 20^{o}C

Ampicillin:

Stock solution 10 mg/ml, final concentration for using is 100 µg/ml

Kanamycin:

Stock solution 10 mg/ml, final concentration for using is 30 µg/ml

Other solutions:

50 mM $CaCl_2$, sterile filtered

Preparation of competent cell:

E.coli strain was directly picked from a frozen stock onto the surface of the LB agar plate, and the plate was incubated overnight at 37 °C. One well-isolated monoclonal cell was picked up, put into 15 ml sterile LB medium and incubated overnight at 37 °C (LB medium without antibiotic). The cells were transferred in 500 ml sterile LB medium to incubate from OD_{600} about 0.02 until to OD_{600} 0.3 at 37 °C and then the cells were centrifuged at 6000 g for 2 min under 4 °C, the pellet was transferred by gentle vortexing into 125 ml ice-cold 50 mM $CaCl_2$ solution. Afterwards the cells were incubated on ice for 20 min and centrifuged again. The pellet was carefully transferred into 25 ml ice-cold 50 mM $CaCl_2$ /10% Glycerol solution by gentle vortexing. Ultimately the suspension was dispensed into small tubes at 100 µl aliquots by shock freezing (through liquid nitrogen) and stored at -70 °C.

2.5.6 Transformation

One aliquot of the competent bacteria was carefully thawed on ice and the ligated DNA was added to the tube of competent cells. The tube was swirled very gently to mix the contents, and it was incubated on ice for 20 min. Afterwards the tube was placed in a preheated water at 42 ^{o}C in the waterbath for exactly 90 seconds, and then it was transferred to ice for 5 min.

The 400 µl preheated LB-medium was added in the tube and it was incubated at 37 ^{o}C for 1 h to allow the bacterial to recover and to express the antibiotic resistance marker encoded by the plasmid. Finally the bacterial was carefully resuspended, and an appropriate volume (about 100 µl per plate) of transformed competent cells was transferred and plated onto the LB-agar plates containing the appropriate antibiotic. The plates were inverted and incubated at 37 ^{o}C overnight. Single bacteria colonies were picked with sterilized toothpicks and put in a bacterial culture-tube containing LB-medium for overnight incubation. The plasmid DNA was finally isolated after

overnight culture. The LB-medium for overnight incubation and in agar plates contained the responsible antibiotics for selection.

2.5.7 RNA isolation

The total RNA isolation was performed with the kit from Qiagen company and all steps were performed with filter-pipette and centrifuged at room temperature. 600 µl RLT buffer containing 6 µl β-mercaptoethanol were added into each sample and mixed. The mixture was carefully pipetted up and down with 2 ml Syringe with 20-gauge needle, the cells were lysed and homogenated. 600 µl 70% ethanol were added and the whole volume was carefully pipetted up and down and 700 µl of the solution were transferred to a Rnaeasy column and centrifuged for 15 sec at 10.000 rpm. The flow through solution was discarded; the rest of 500 µl mixes were transferred to the same Rnaeasy column and centrifuged for 15 sec at 10.000 rpm once, the flow through solution was discarded. Afterwards 350 µl RW1 buffer were added in column and the samples were centrifuged again and the flow through solution was discarded. 500 µl wash buffer RPE were added into the column and the samples were centrifuged for 15 sec at 10.000 rpm, the flow through solution was discarded, the wash step was repeated once. New 1.5 ml tube was used to collect the RNA solution, while 30 µl Rnase free water was added into the column and centrifuged for 1 min at 10.000 rpm. The RNA samples were immediately frozen at - 80 °C for analysis.

2.6 Ecdysone mammalian expression system

The ecdysone-inducible mammalian expression system (Invitrogen, Karlsruhe, Germany) is based on the ecdysone-system from Drosophila. In this expression system both subunits of a heterodimeric ecdysone receptor are constitutively expressed in the regulator vector pVgRXR. The ecdysone-responsive promoter, which finally drives expression of the gene of interest, is located on the pIND expression vector. The ecdysone system expresses the gene of interest by using the ecdysone-analog ponasterone A. The target gene was first cloned into the inducible expression plasmid pIND (SP1), and this construct was stably transfected into EcR293 cell lines by Lipofectmine 2000 (Clontech, Germany) according to the standard transfection protocol. These EcR293 cell lines expressed stably the heteromeric ecdysone receptor. The cDNA of NOS2 (inducible nitric oxide synthase) containing the entire open

reading frame, and partial 5' and 3' untranslated sequences of NOS2 were cloned into an ecdysone inducible mammalian expression vector, pIND(SP1)/Neomycin (Invitrogen Corp, CA, USA). The stable transfected cells were selected in medium containing both G418 (400 ng/ml) and Zeocin (400 µg/ml). The clones growing up after about 4 weeks of selection were picked up and further analysed.

2.7 Cell biological methods

2.7.1 Cell culture

Medium:

DMEM (Dulbecco's modified Eagle's medium) GibcoBRL/Invitrogen, Karlsruhe
Without sodium pyruvate
With 4500 mg/ml glucose
With pyroxidine HCl

For cell culture, the following solutions were added:

10% FCS
1% P/S
1% L-Glutamine
1% Hepes

Solutions or reagents were used in cell culture:

Hepes Buffer 1 M BioWhittaker Europe, Vervies Belgien
In 0.85% NaCl

Fetal Bovine Serum (FBS) BiochromKG, Berlin

Penicillin-Streptomycin GibcoBRL/Invitrogen, Karlsruhe
10.000 U/ ml penicillin
10.000 µg/ml streptomycinsulfate in 0.85% NaCl

L-Glutamine PAA, Cölbe
200 mM

Gentamicin-ratiopharm 40 SF Tatiopharm Ulm

200 mM

Trypsin/EDTA PAA, Cölbe
0.1% Trypsin for EcR293 and EcR293-NOS2 cell lines

G418 BibcoBRL/ Invitrogen, karlsruhe
Genticin G418 sulfate 10 mg/ml

Zeocin Invitrogen, Karlsruhe
100 µg/ml

Cell lines:

HEK-293 (human embryonic kidney cell)

- HEK-293 cell lines were developed from human embryo kidney cells and immortalized by adenovirus transfection
- This cell line is adherent and showed fibroblastic morphology.
- This cell line has high transfection efficiency and is convenient for transient or stable transfection of protein to analyze.

EcR293

-From Invitrogen, Karlsruhe, Germany; developed from HEK-293
- This transgene cell lines expressed stably the modified ecdysone-receptor and was used in ecdysone-system for the pIND (SP1)-regulatable stable expression.
- Under Zeocin selection (400 µg/ml)

EcR293-NOS2 cells

This cell line was developed from EcR293 with stable transfection of the target construct containing NOS2 gene: pIND-NOS2-cds
Selection medium: Zeocin 400 µg/ml and G418 400 µg/ml

Culturing of the cells

DMEM (for EcR293, EcR293-NOS2 cells)
10% FBS

1% L-Glutamin 200 mM
1% Hepes Buffer 1 M
1% penicillin/Streptomycin 10mg/ml

The cells grew adherently and were cultured in the incubator at 37°C with 5% CO_2 atmosphere. After incubation for 2-3 days, confluent cultures were trypsinized, diluted and seeded again. For trypsinizing the cells, the medium was removed and the cells were washed one time with PBS, and 1 ml 0.1% trypsin/EDTA was put onto the cells, incubated for 3-5 min at room temperature. Then the trypsin was removed and the cells were incubated at 37 °C for 5-7 min. The cells were resuspended in 5 ml medium in a 15 ml tube. Afterwards the cells were very carefully pipetted up and down for several times. From this cell suspension, 0.5 ml cell solution was transferred into a new cell culture plate with addition of 10 ml medium containing responsible antibiotics (Zeocin and G418). Finally the cells were cultured at 37 °C in the incubator.

2.7.2 Conservation of the cells

The medium for cell conservation:
20 % FBS
10 % DMSO
70 % medium

While the growing of 70% confluency in 10 cm dish was achieved, the cells were washed one time with PBS (without EDTA), and then trypsinized by 0.1% trypsin/EDTA. After trypsinization, the cells were harvested in 10 ml medium, and centrifuged at 1000 g for 5 min. The supernatant was removed and the cell pellet was resuspended in 5 ml cell conservation medium. The tubes containing cells were carefully placed in a Styropor-box, and it was placed into -70 °C. For long-term conservation of the cells, the tubes should transfer into liquid nitrogen for storage.

For the reculture of the cells, one aliquot of the frozen cells was taken from -70 °C or liquid nitrogen and immediately thawed at 37 °C in the waterbath. Then they were resuspened in 10 ml medium and centrifuged at 1000 g for 5 min. This step removed

the DMSO, which had been added in the medium for freezing the cells. The cell pellet was resuspended in 10 ml medium and they were seeded in cell-culture dishes at 37 °C.

2.7.3 Transfection

Transfection Solution

-Lipofectamine 2000 BibcoBRL/Invitrogen, Karlsruhe, Germany

-DMEM without serum
-DMEM without antibiotic

To verify the inducible expression of the NOS2 gene, transient transfection was performed and the expression was detected by Western blot. One day before the transfection, the cells were seeded in 6-well plate about 50% confluency, and they were cultured with DMEM containing 10% FCS, 1% Hepes, and 1% L-Glutamine and without any antibiotics. For the transfection 5 µg DNA and an equal volume of Lipofectamine 2000 (here 5 µl) for the single well were diluted respectively into 250 µl DMEM without serum and antibiotic, the suspension were incubated at room temperature for 5 min.

The diluted DNA and Lipofectamine 2000 solution from the above step were carefully mixed together and the solution was incubated at room temperature for 20 min. After incubation 100 µl of the complexes was carefully added directly into each cell well, and mixed very gently by rocking the 6-well plate. The DMEM medium with serum and antibiotic was replaced after 8-10 h. The cells were incubated at 37 °C in 5% CO_2 for 48 h until analysis of the target gene expression was performed.

2.7.4 Western blot

2.7.4.1 Western blot solutions

50X protease inhibitor solution Roche, Mannheim, Germany

Benzonase Roche, Mannheim, Germany

1X PBS/Tween

1X PBS / 1% Tween 20

2X sample buffer

Tris-HCl pH 6.8	120 mM
Glycerin	10%
SDS	4%
Beta-mercaptoethanol	4%
Bromphenolblau	0.02%

Acrylamide solution	(37.5:1)
Acrylamide	37.5%
Bis-Acrylamide	1%
In H_2O	

resolving gel (8%)		**resolving gel (10%)**	
Acrylamide solution	10.7 ml	Acrylamide solution	10.7 ml
1.8 M Tris-HCL pH 8.8	8 ml	1.8 M Tris-HCL pH 8.8	8 ml
H_2O	20.8 ml	H_2O	20.8 ml
10% SDS	400 µl	10% SDS	400 µl
10% ammoniumpersulfat	200 µl	10% ammoniumpersulfat	200 µl
TEMED	8 µl	TEMED	8 µl

Stacking gel

Acrylamid solution	3 ml
0.8 M Tris-HCL pH 6.8	4 ml
H_2O	12.7 ml
10% SDS	200 µl
10% ammoniumpersulfat	100 µl
TEMED	10 µl

Running buffer (10 X Laemmli-buffer)

Tris	30.25 g
Glycin	144 g

SDS 10 g

H_2O add until to 1 L

2.7.4.2 SDS-polyacrylamid gel electrophoresis (SDS-PAGE)

SDS-PAGE was used to separate the proteins into their individual polypeptide subunits and minimize their aggregation in an electrophoresis field. After the protein was treated by the ionic detergent SDS, reduced with beta-mercaptoethanol and denatured by heating at 99 °C, the protein was separated by gel electrophoresis based on the different molecule mass of the polypeptide. Through using protein of known molecular mass, it was possible to elevate the molecule mass of the polypeptide chains. The SDS-PAGE was performed with a discontinuous buffer system. This PAGE buffer system was modified by Laemmli and Andrews (Laemmli, 1970; Andrews, 1986), besides the gels used components, 0.1 % SDS is added. Here 8 % or 10 % acrylamide in the experiments were used. The gels were used directly or stored under humid conditions at 4 °C until to use.

2.7.4.3 Samples preparation for Western blot

For the preparation of samples for Western blot, the medium was removed and the cells were washed one time with 1X PBS. Then the cells were harvested in 100 µl (for one well in 6-well plate) NP-40 solutions (1% NP-40 in 1X PBS containing 1 X protein inhibitor), and the solution containing cells was immediately incubated on ice for 15 min for cell lysis. Afterwards it was centrifuged at 1000 rpm for 10 min. Then the protein concentration was determined, and 30 µg or 40 µg proteins was mixed with 2x loading buffer, and the samples were heated at 99 °C for 5 min, then the samples were put on the ice. The samples and responsible molecular marker (SDS7B2, Sigma) were loaded on the SDS-PAGE gel. The samples were run at 25 mA/gel in stacking gel and 30 mA/gel for separation under room temperature.

2.7.4.4 Blotting

Solutions:

Blot solution:

Stock solution for blot buffer I and II:

1M Tris-HCl pH 10.4

H_2O add distilled water until to 1 L

Blot buffer I:

Stock solution	300 ml
H_2O	700 ml

Blot buffer II:

Stock solution	25 ml
H_2O	975 ml

Blot buffer III:

E-amino-n-corporic-acid	5.24 g
H_2O (distilled)	until to 1 L

The gel containing proteins was transferred on the PVDF-membrane using the semi-dry method as follows:

-Anode
-3 pieces of Whatman papers, with buffer I
-3 pieces of Whatman papers, with buffer II
-PVDF-membrane, first in methanol, then in water, finally in buffer II
-gel
-6 pieces of Whatman papers, with buffer III
-Cathode

The PVDF-membrane (Millipore, Schwalbach) was activated in methanol, washed in H_2O and finally wetted in blot buffer II. The protein was transferred on the PVDF-membrane in a Multiphor II-Kammer instrument (Millipore, Schwalbach) according to the manual. The protein was transferred from the gel to PVDF membrane at 0.8 mA/ cm^2 gel area at room temperature for 90 min.

2.7.4.5 Immune detection of the protein in Western blot

Buffers:

I-Block solution:

1 g I-Block reagent in 500 ml 1 X PBS and incubated at 60 °C for 2 hours, afterwards filled in 500 µl Tween.

Wash buffer:

1 x PBS /Tween

Block buffer:

0.2 % I-Block™ reagent

Assay buffer:

Diethanolamin 0.1 M, $MgCl_2$ 1 mM

CDP-Star detection system:

Nitro-Block: 1:20 in assay buffer

CDP-Star: 1:200 in assay buffer

After blotting, the PVDF membrane was washed about 2-5 min in wash buffer and then incubated in I-block buffer for 1 h at room temperature, and it was incubated with the first antibody in I-block buffer at 4 °C overnight. Afterwards it was washed 3 times by wash buffer respectively for 5 min, and following this step the membrane was incubated with second antibody at room temperature for 1 h. Then it was washed 3 times (each for 20 min) by wash buffer and incubated with assay buffer for 2 times (each for 10 min). Finally the PVDF membrane was incubated with Nitro-Block (2 ml / blot) buffer and 5 min with CDP-Star buffer. The membrane was carefully covered with a transparent film and the luminescence development was performed from 1 min to 1 h on the film in an autoradiography box.

2.7.4.6 Preparation samples of cytoplasmic and nuclear extracts for Western blot

(Modified method from Schreibert and Schaffner, 1989)

Extraction buffer A:

10 mM HEPES pH7.9

10 mM KCl
0.1 mM EDTA
0.1 mM EGTA
1 mM DTT
0.5 mM PMSF

Extraction buffer B:
20 mM HEPES pH 7.9
0.4 M NaCl
1 mM EDTA
1 mM EGTA
1 mM DTT
1 mM PMSF

Other solutions:
10% NP-40 in PBS
1 X protease inhibitor solution
2X sample buffer

After culture of the transfected cells for 48 h, the medium was removed, and the cells were washed one time with PBS, and were then harvested in 400 µl cold extraction buffer A. Typically the number of the cells was between 0.5-1 x 10^6 cells. The cells were gently pipetted in a yellow tip several times. Then the cells were allowed to swell on ice for 15 min. Afterwards 25 µl of a 1% solution of NP-40 (with 1 X protease-inhibitor) was added and the samples were vigorously vortexed 10. The homogenates were centrifuged for 30 seconds. The supernatant was carefully transferred to a fresh tube and stored at - 20 ^oC for future analysis.

The nuclear pellet was resuspended in 50 µl ice-cold buffer B, and the samples were vigorously rocked at 4 °C for 15 min on a shaking platform. Afterwards the nuclear extract was centrifuged at 4 ^oC for 5 min in a microfuge, and the supernatant was transferred to a fresh tube and the nuclear extract was stored at - 20 ^oC for analysis.

30 µg proteins of each sample were mixed with 2X sample buffer, and they were heated at 99 °C for 5 min, then the samples were transferred on ice for cooling down. Afterwards the samples were loaded on a SDS-PAGE gel for analysis.

2.7.5 Immunofluorescent staining

Fixation solution:

PFA 4% paraformaldehyd in PBS

Methanol/Acetone mixed at ratio 1:1

Wash buffer:

0.1% Triton-X-100 in PBS

Block solution:

3% BSA (Albumin Bovine Fraction V)

0.1% Tween

In PBS

Dye stuff:

Hoechst dye

2.7.5.1 Preparation of the cells for immunostaining

The EcR293-NOS2 cell lines were seeded in one 8-chamber slide (10.000 cells / chamber) or one 6-well plate (in this plate containing cover slip) ($1X10^5$ cells/well). The cells were induced by ponasterone A at the appropriate concentration.

2.7.5.2 Immunofluorescent staining

After 48 h induction by ponasterone A, the cells in 6-well plate or 8-chamber slide were washed twice with PBS. Then they were fixed with PFA solution and incubated at room temperature for 10 min, or alternatively the cells were also fixed with methanol/acetone, and incubated at -20 ^oC for 5 min, and then allowed to dry at - 20^oC.

The cells were washed three times with wash buffer, and afterwards the cells were incubated in block solution at room temperature for 1 h. The first antibody was diluted in block solution at 1:200 and the cells were incubated with first antibody for 2 h in a

humid chamber. The cells were washed again with wash buffer 3 times, and they were treated with second antibody (which was diluted in block solution at 1:200) at room temperature for 2 h. The cells were washed 3 times with wash buffer, and washed 2 times again with normal PBS, afterwards the cells were incubated with Hoechst dye (1:10.000 in PBS) at room temperature for 5 min in the dark.

The cells were washed twice with normal PBS, and mounted with permafluor medium, and dried overnight. Finally the staining was analysed by confocal laser scanning microscope (Zeiss 510-UV, Zeisse, Germany).

2.7.6 Detection of the cell viability

After the treatment with anti-APO-1, the detection of the cell viability was measured in 96-well plates by CellTiter-Glo Luminescent Cell Viability Assay (Promega, Mannheim, Germany) or MTT assay (Sigma, USA).

2.7.6.1 Preparation of the cells

The EcR293-NOS2 cell clones were seeded at the confluency of 15.000 cells/well in 96-well plates. After induction by ponasterone A at 5 μM for 24 hours, the cells were treated with 200 ng/ml anti-APO-1 for another 24 hours, after treatment the cells were harvested for CellTiter-Glo assay or MTT measurement.

2.7.6.2 CellTiter-Glo luminescent cell viability assay

The CellTiter-Glo assay is based on the quantification of the ATP present, which signals the presence of metabolically active cells. The assay was followed as recommended in the manual from the company. The first step was addition of CellTiter-Glo reagent (Promega, Mannheim, Germany) into the medium, which contained cultured cells. The CellTiter-Glo reagent contained Luciferase, Mg^{2+}, and Luciferin, the enzyme Luciferase was oxidized in the presence of Mg^{2+}, ATP and Luciferin. After this reaction the light was emitted. The additions of reagent resulted in cell lysis and lead to generation a luminescent signal proportional to the metabolically active ATP present. The amount of ATP is directly proportional to the number of cells presenting in culture. The luminescent signals produced by the reaction have a half-life of more than 5 h, and it was measured using a luminometer.

2.7.6.3 MTT measurement

Cell survival after treatment with anti-APO-1 was detected by the MTT-cytotoxicity assay as modified by Mosmann on 1983. MTT (3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium Bromide) is a water soluble tetrazolium salt. It will form a violet, insoluble crystal form in inner mitochondrial by reduction through dehydrogenase in the mitochondria. The measurement is based on the dye conversion reaction from MTT, which can be observed in all viable cells. However dead cells do not this conversion and remain colorless. For measurement, the MTT stock solution should be freshly added at a concentration of 8 mg/ml in PBS (before using the solution, it should be first sterile filtered to eliminate insoluble particles). The MTT solution was added at a ratio of 1:10 in the cell culture medium, and incubated at 37 °C for two hours. At the end of incubation, the cell culture medium was carefully removed, and the intracellular formazan crystal was immediately filled with MTT stop solution. After the lysis, the quantification of the dye intensity were directly measured with a SPECTRA FluorPlus-Fluoresence Reader at the 570 nm main wavelength and 630 nm reference wavelengths for subtraction of background. The data measurement from SPECTRA FluorPlus-Software was saved directly in Excel-data tables and analyzed.

2.7.7 Griess assay

Griess buffer:

1X 0.1% naphthylethylenediamine dihydrochloride in distilled water plus 1X 1% sulfanilamide in 5% concentrated H_3PO_4

Other solution:

Stock solution: $NaNO_2$ 10 mM

100 μM $NaNO_2$ is prepared for use

2.7.7.1 The basis of Griess assay

The Griess assay is one of the methods to detect quantitatively the biological reaction products of nitrite (NO_2^-), this detection is achieved through a red azo-dye, which exhibites a maximum absorption at 540 nm after reacting with nitrite. Because nitrite (NO_2^-) is a stable degradation product from nitric oxide (NO), the measurement of

nitrite concentration can be used as a method or index to indirectly analyze the concentration of NO.

2.7.7.2 Performance of Griess assay

The cells were seeded in 6-well plates and cultured with DMEM medium without phenol red. The cells were induced with ponasterone A at 5 µM concentration for 48 hours. The medium was collected and the cells were harvested by 1 % NP-40 solution (containing 1 x protein inhibitor). The protein concentration of each sample was determined by standard method. For measurement of nitrite concentration in the medium, the following steps were carried out:

- prepare the standard curve

Tube (standard con.)	µl of 100 µM $NaNO_2$	µl of distilled water
Nr. 1 (0 mM)	0	300
Nr.2 (1 mM)	3	297
Nr.3 (5 mM)	15	285
Nr.4 (10 mM)	30	270
Nr.5 (15 mM)	45	255
Nr.6 (20 mM)	60	240

Con.= concentration

300 µl solution from each sample was taken and 60 µl Griess buffer was added in each standard sample and sample. They were centrifuged at 3500 rpm and 4 °C for 15 min. Then 300 µl standard samples and samples were transferred in 96-well plate (transparent type) and the absorbance was measured at 570 nM

Comparing the absorbance of cell samples and the standard samples, calculating the respondent concentration of $NaNO_2$ for samples, finally the concentration of nitrite generated from NO in the medium should be presented in the formal "µM (nitrite) /µg protein".

2.7.8 Caspase activity analysis

Caspase activation was measured through commercial assay kit, Caspase Assay Fluoremetric Kit from Biosource for caspase-3, caspase-4, caspase-6, caspase-8 and caspase-9. The assay is based on the proteolytic cleavage of a peptide substrate for a particular caspase into a fluorescent product.

The caspase assay kit contained buffers and peptide substrates for this analysis. The substrates contain on the fluorescent dye, 7-amino-4-trifluoromethylcoumarin (AFC). The substrates were synthesized by coupling the carboxy group of an amino acid derivative or a peptide of a specific sequence to the amine groups of the dyes. Before proteolytic cleavage, the substrates have no or very low fluorescence. After the caspase cleavage, the dyes are released and an increase in the fluorescence can be measured. The AFC-based substrate generated a fluorescent product (AFC), that has a long emission (505 nm), which can be quantified with the SAFIRE Doppel-Monochromator instrument. The fluorescent intensity data was saved on the computer and later analyzed using Excel software.

2.7.8.1 Preparation of samples for caspase assay

EcR293-NOS2 cells were seeded in 6-well plates about 50% confluency, and the cells were induced by ponasterone A at 0, 0.5, 1, 3, 10 μM for 24 hours, and then the cells were treated by anti-APO-1 at the concentration of 350 ng/ml for another 24 hours. After 24 hours treatment by anti-APO-1, the medium and the cells were harvested into 15 ml tubes. They were centrifuged and resuspended in 100 μl 1% NP-40 solution and afterwards they were incubated on ice for 15 min, the samples were centrifuged once again. The supernatant were used for protein concentration determination.

2.7.8.2 Caspase activity assay

Cell lysis buffer in caspase activation assay

20 mM Tris/HCL pH 8.0
5 mM EDTA
0.5% Triton X-100

For the lysis of the cells, the protease-inhibitor (in the ratio of 1:25) was freshly added in the above lysis solution.

2X reaction buffer

50 mM HEPES
100 mM NaCl
20% Glycerol
0.1% CHAPS

Substrates

Caspase -3: Ac-DEVD-AFC
Caspase -4: Ac-LEVD-AFC
Caspase -6: Ac-VEID-AFC
Caspase -8: Ac-IETD-AFC
Caspase -9: Ac-LEHD-AFC

Other solution:

10 mM DTT

After determination of the protein concentration, 40 µg proteins were taken for the activity assays of caspase-8, caspase-9 and caspase-6 and 20 µg protein as taken for the assay of caspase-3 and caspase-4 activation. The appropriate volume of the samples was pipetted into Eppendorf tube and filled with lysis buffer until the total volume was 50 µl. In the tube another 50 µl 2X reaction buffer/10 mM DTT were added. Afterwards 5 µl caspase substrate was pipetted into. For control, lysis buffer and 2X reaction buffer were pipetted with and without substrate respectively. The samples were incubated at 37 °C for 2 h under the darkness.

80-90 µl of the incubated samples were pipetted into the black plate, and the fluorescent intensity was measured at the wavelength 405/535 nm on the SARIRE Doppel-Monochromator. The data were saved on the computer and analysed.

2.7.9 JC-1 staining for change of mitochondria membrane potential

JC-1 : 5,5', 6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazoly-lcarbocyanine chloride
JC-1 is diluted in DMSO, stored at 4 ^{o}C
For the staining JC-1 was freshly diluted in 100% ethanol

2.7.9.1 JC-1 introduction

JC-1 is a specific cationic dye for mitochondria membrane potential and has exhibits membrane potential-dependent accumulation in mitochondria after staining mitochondria in living cells. JC-1 monomer is in equilibrium the so-called J-aggregates, which are favored at higher dye concentration or higher mitochondria membrane potential. The monomer JC-1 has green fluorescence at 527 nm light wave length, while the J-aggregates have red fluorescence at 590 nm wave length.

After JC-1 staining of mitochondria, it shows a fluorescence emission shift from green at 525 nm to red at 590 nm by FACS analysis procedure. The depolarization of mitochondria membrane is indicated by a decrease in the red fluorescence intensity. In healthy cells, the dye stains mitochondria as red, while in apoptotic cells, mitochondria membrane potential become loss, and the JC-1 cannot accumulate within mitochondria, therefore in these apoptotic cells, JC-1 remains in a green fluorescent monomeric form. The change of intensity from red to green fluorescence in cells is used to measure the change of the mitochondria membrane potential and can be detected by FACS analysis.

2.7.9.2 Preparation of cells for JC-1 staining and measurement on FACS

The cells were seeded in 6-well plate about $4x10^5$ cells/well, and they were induced by ponasterone A at a concentration 0, 0.5, 1, 3, 10 µM for 24 hours, and then the cells were treated with anti-APO-1 350 ng/ml for another 24 h. Afterwards the medium was collected in 15 ml Falcon-tube, the cells were trypsinized, transferred and taken with collected medium. The mix was centrifuged at 500 g for 5 min. The supernatant was removed and the cell pellet was diluted in 200 µl medium.

JC-1 was diluted in 70% ethanol by 1:10 (here 3 µl JC-1 in 27 µl 70% ethanol). Then 4 µl JC-1 solution was added in each 200 µl cell sample, and mixed very gently. They

were incubated at 37 ^{o}C for 20 minutes in the dark. Following the cells were washed with PBS and centrifuged once at 500g for 5 min. The supernatant was removed, the cell pellet was resuspended in 500 µl PBS and they were immediately measured by FACS. The data was analysed with the software Cellquest-Pro.

2.7.10 Flow cytometry (FACS) analysis

FACS buffer

PBS
0.4 % BSA
0.03 % NaN_3
10 mM EDTA
20 mM HEPES
Sterile filtered

FACS (fluorescence-activated cell sorter) analysis is a method to quantify and characterize the cells. Through fluorescent markers (fluorescently labeled monoclonal antibodies) staining on the cells, FACS machine can recognize and sort the cells. FACS machine possess the capacity of performing multiparameter analysis on a single cell, such as size, volume, viscosity, DNA or RNA content and surface antigens. Information obtained from FACS analysis is variable, depending on the properties which are analysed. Normally the data is primarily displayed as a form of histogram or plot. The x-axis of the histogram shows the fluorescent intensity and y-axis displays the number of cells found within each parameter. When analyzing more parameters, the histogram is displayed in the multi-dimensional color coordinated display.

2.7.10.1 Cell cycle-profiles and determining of apoptosis by the FACS analysis

For the FACS analysis, the cells were stained by propidium iodide (PI). Propidium iodide interacted with DNA in pre-fixed cells. It was activated to emit fluorescence under the 480 nm laser source, and produced an emission signal at 650 nm. In flow cytometry analysis, the normal growing cells show a typical DNA-profile from G1, G2 and S phase after propidium iodide staining. When the cell cycle is influenced or disrupted (for example during apoptosis), and the change in DNA-profiles will be observed according to the measurement of the peaks from G1, G2 and S-populations.

DNA will become fragmented during the apoptosis process. Reduction in the DNA content of the stained cells reveals the apoptotic cells in the sub-G1 population determined by FACS analysis.

2.7.10.2 Prepare the cells for apoptosis measurement by the FACS analysis

PI-staining solution:
50 µg/ml propidium iodide in PBS

Cells were seeded at a confluency 150.000 cells/well in 12-well plates, and the cells were induced in medium by ponasterone at 5 µM. After 48 hours culture, the cells were harvested for the analysis.
For analysis of CD95- or TRAL-mediated apoptosis, after induction by ponasterone for 48 h, the cells were treated with anti-Apo-1 or TRAIL. Then the cells were trypsinized, washed with PBS and centrifuged at 1000 rpm for 5 min. The cell pellet were resuspended in PBS and it was fixed with pre-cooling 100 % ethanol at -20 °C for half hour. The fixed samples were centrifuged again and the pellet was resuspended in 500 µl PBS containing staining PI solution. The stained samples were kept at 4 °C overnight.

2.7.10.3 FACS measurement for apoptosis

The analysis of fluorescence can be carried out by one FACSCalibur instrument (Becton-Dickinson, Heidelberg, Germany). The 488 nm Argon-laser was used and the fluorescence was measured. The data were directly shown on the screen in a histogram form, and 10.000 cells were used to measure. All data were registered, saved and analysed under the CellQuestPro-software.

2.7.10.4 The expression of CD95 receptor or TRAIL receptor analysed by FACS

For the quantification of CD95 or TRAIL receptor on the extracellular membrane, the specific antibody for CD95 or TRAIL receptor was used and it was stained by fluorescent-labeled second anti-body R-Phycoerythrin.

- Preparation the cells for CD95- or TRAIL- receptor measurement on FACS

The EcR293-NOS2 clones were seeded in 12-well plates and induced by ponasterone at the concentration of 0, 0.5, 1, 3, 5, 10 µM for 48 hours. Following the induction, the cells were trypsinized ,centrifuged and resuspended in 100 µl medium containing responsible anti-bodies (for CD95 receptor using anti-APO-1, for TRAIL receptor using anti-DR4 and anti-DR5 antibodies), they were incubated at room temperature for 15 min. Then they were centrifuged, washed one time in medium. The cells were resuspended and incubated at room temperature for 15 min in 100 µl medium including fluorescent-labeled second antibody. After the incubation by second anti-body, the cells were washed one time by PBS; they were resuspended in 500 µl PBS and immediately measure them on the FACS.

- Measurement of receptor staining on the FACS

The measuring process was carried out same as the steps mentioned above. The second antibody R-Phycoerythrin will be activated at 488 nm and fluoresced at 575 nm. The intensity of the fluorescence of the stained cells corresponds to the expression of the receptor on the extracellular membrane surface and the number of the CD95-receptor positive cells. When the expression of the CD95 or TRAIL receptors is increased, the fluorescent peak of the FACS-analysis will responsibly move towards right on the histogram comparing with the control. Mouse IgG3 isotype was used for this analysis.

2.7.11 Chrome assay of T cell killing

An allo-reactive A2.1-specific polyclonal CTL (CTL CD8 Allo-A2) were established as reported (Theobald, 1995) for the Flu M1 58-66 peptide (CTL CD8x A2K^b FluM1) has been described previously (Drexler, 1999). CTLs were cocultured with ^{51}Cr labeled EcR293-NOS2 cells at the indicated effectors to target (E:T) ratios. ^{51}Cr release was measured after 4.5 h. The peptide specificity of the CD8 x A2K^{b}FluM1 CTLs were controlled by their failure to the lysis of the target cells loaded with an irrelevant peptide.

3. Results

3.1 Establishment and verification of NOS2-expressing clones

3.1.1 Cloning

To investigate the physiological and pathological functions of endogenously produced nitric oxide (NO) in the cells, an inducible mammalian expression system, namely ecdysone expression system, was used. This system allows induction of a target gene by ecdysone or its analog, ponasterone A. The first step was cloning the NOS2 gene into pIND(SP1). The NOS2 gene fragment was obtained from pTRE-NOS2-cds construct. First, the NOS2 gene was cloned in the pBluescript SK(+/-) vector to flanking obtain restriction enzyme sites in the multicloning site that are compatible with the pIND plasmid.

The cloning procedure of pIND (SP1)-NOS2:

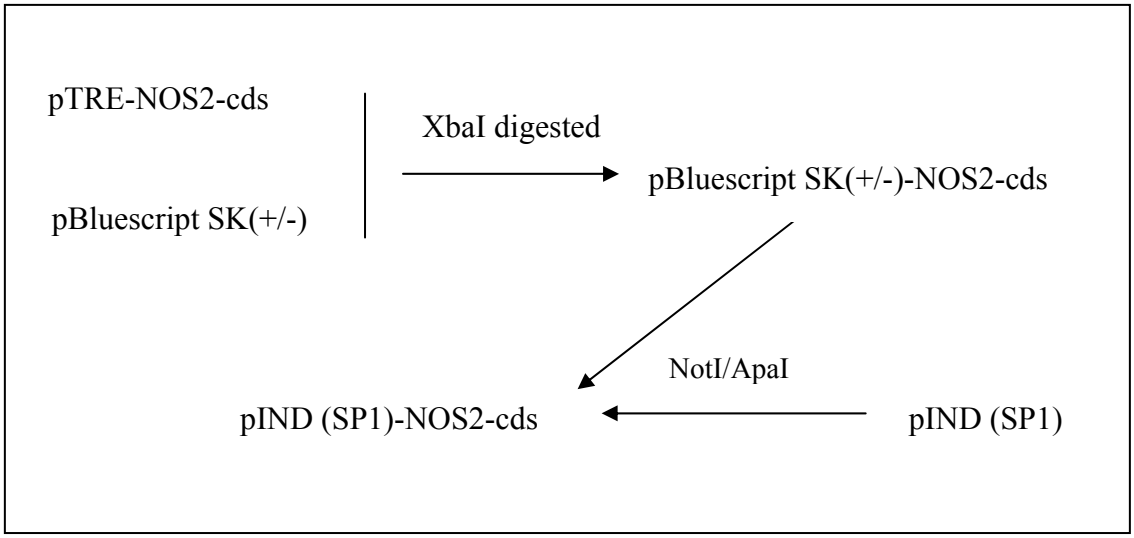


Figure 1: Overview about the strategy of cloning pIND-NOS2 for transient and stable transfection.

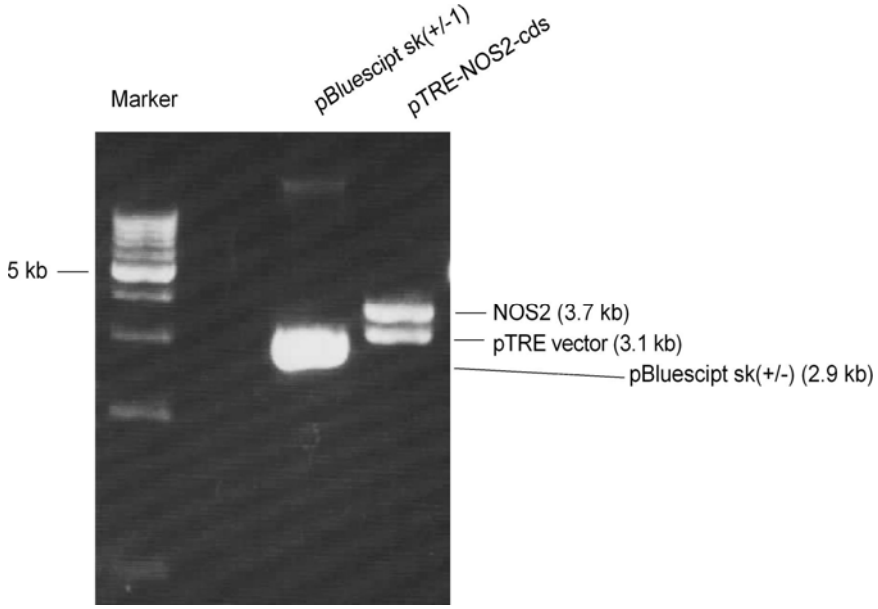
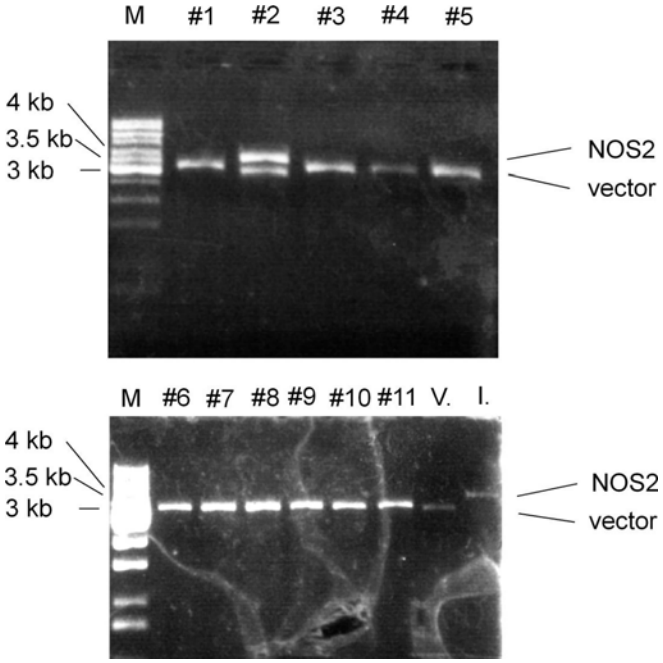


Figure 2: Agrose gel analysis of digestion for pBluescript and pTRE-NOS2-cds by XbaI

The pTRE-NOS2-cds plasmid was digested by XbaI producing a 3.7 kb human NOS2 fragment and a 3.1 kb of pTRE vector fragment. The pBluescript SK(+/-) was also digested by XbaI producing a 2.9 kb linear DNA (Figure 2). The NOS2 gene fragment was purified by phenol-chloroform after performing gel extraction and was ligated in the XbaI-digested pBluescript SK (+/-) vector.

DNA mini-preparation was performed after the bacteria transformed by the pBluescritp SK(+/-)-NOS2 construct. The DNA samples from different minipreps were digested by XbaI and analysed on 0.8 % agarose gel (Figure 3A). For the DNA orientation analysis, the DNA was digested by EagI, the result was shown in Figure 3B.

A.



B.

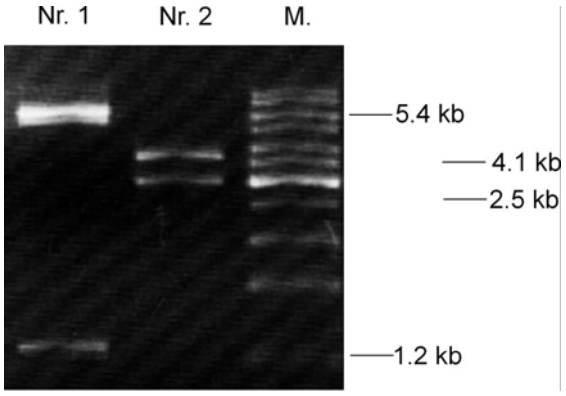


Figure 3: NOS2 fragment and vector were analysed by agarose gel electrophoresis. 11 DNA samples from different minipreps were digested (from #1 to #11). V. and I. represents vector alone and insert alone respectively for control in Figure 3A. M represents DNA marker. Figure 3B showed the analysis of DNA orientation. DNA fragments were digested by EagI, and the right orientation will be from 4.5 and 1.2 kb DNA fragment, the wrong orientation will be composed by 4.1 and 2.5 kb fragments.

The purified pBluescriptSK-NOS2 and pIND(SP1) vector were digested by NotI/ApaI. The NOS2 fragment produced from pBluescriptSK-NOS2 was ligated in the NotI/ApaI digested pIND(SP1) vector for producing pIND-NOS2 construct.

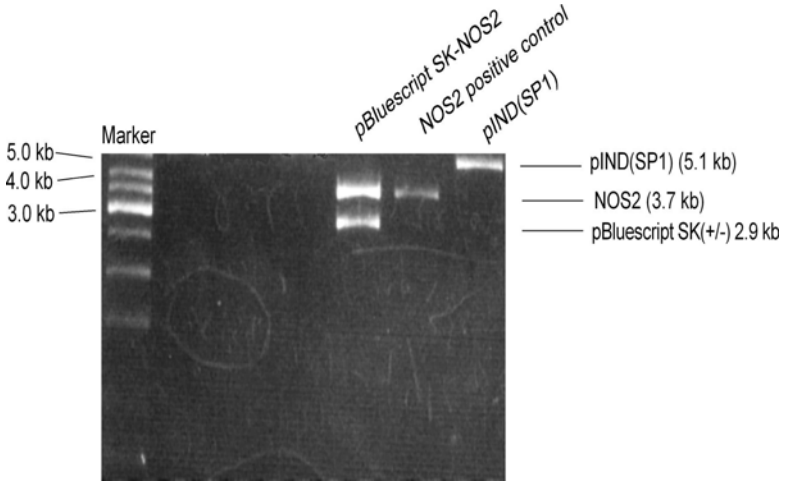


Figure 4: The digestion results from pIND(SP1) vector and pBluescriptSK-NOS2 analysed by agarose gel.

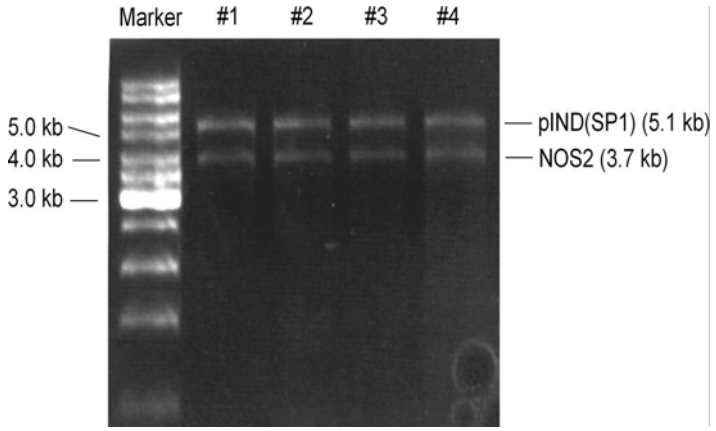


Figure 5: The ligated pIND-NOS2 construct was digested by NotI/ApaI and analyzed on agarose gel. Here shows the digestion from the part of the different samples (from #1 to #4). pIND(SP1) is about 5.1 kb and NOS2 fragment is about 3.7 kb.

The results in Figure 5 show that NOS2 fragment was correctly ligated in pIND(SP1) vector for producing pIND-NOS2 plasmid. The construct was sequenced to ensure the coding sequence contained no errors.

3.1.2 Analysis of transient expression

3.1.2.1 Immunostaining for transient expression of NOS2

After construction of pIND-NOS2 plasmid, NOS2 expression was controlled by transferring it into EcR293 cells.

The recombinant construct pIND-NOS2-cds was transfected into EcR293 cells according to the standard method by Lipofectamine 2000 transfection reagents. The transfection was performed in the 6-well plates. Before the transfection, one cover slip was placed in each well of the plates. The EcR293 cells were seeded about 60-70 % confluency in the plates, and 4 µg DNA was used for transfection in each well. After the transfection, the cells were cultured at least 8 h, and then induced by addition of ponasterone A at 5 µM concentration. The induced cultures were kept for an additional 48 h and then the cells were carefully washed by PBS and immunostained. NOS2 antibody and a fluorescent-labeled secondary antibody were used for this staining. After secondary antibody treatment, the samples were incubated with Hoechst dye for 5 min before washing and mounting.

The immunostained samples were examined using a laser scanning microscope and the images were saved in Zeiss software database before export.

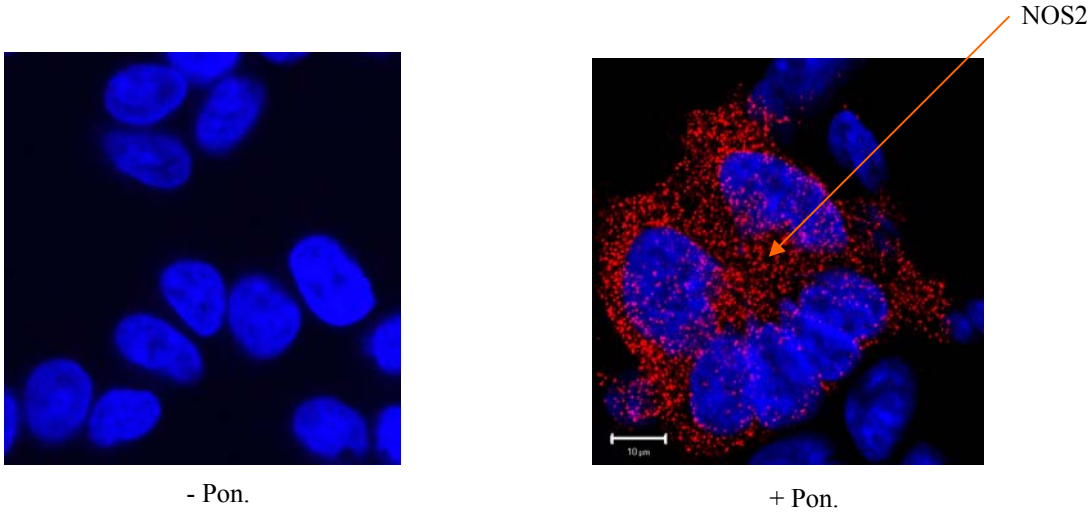


Figure 6: Immunofluorescent staining of NOS2 expression in EcR293 cells after transient transfection. Cells were not induced (- Pon) or induced by 5 µM ponasterone (+ Pon), the cells were stained for the analysis of the NOS2 expression; the cells were imaged by a Zeiss LSM 510 UV laser scanning microscopy. Nuclei are stained in blue. Bar in low panel depicts 10 µm.

Figure 6 shows the expression of NOS2 in EcR293 cells after transfection. The cells were not induced (- Pon.) or induced (+ Pon.) by ponasterone A. From the figures, in the uninduced cells (- Pon.), the expression of NOS2 were rarely observed, but in the induced cells (+ Pon.) the expression of NOS2 was observed in the cytosol in a number of cells (red region). These results demonstrated that the expression of NOS2 was inducible by ponasterone A and the localization of NOS2 proteins was mainly in the cytosol of the cells.

3.1.2.2 Analysis of NOS2 expression in transient transfection by Western blot

Western blot was performed to verify the transient expression of NOS2 in EcR293 cells after cloning of pIND-NOS2 plasmid. EcR293 cells were seeded in then 6-well plates and the plasmid pIND-NOS2 was transfected into cells with Lipofectamine 2000 reagent. 4 µg DNA was used for each well of transfection. After transfection the cells were induced by ponasterone A at 5 µM concentration for 48 h. The cells were washed by PBS and harvested directly with 1 % NP-40 solution. 40 µg protein was used and mixed with 2 x loading buffer, and finally loaded on the 8 % SDS-PAGE gel to run. The rabbit anti-NOS2 polyclone antibody (from Santa Cruz Corporation, USA) was used for this analysis.

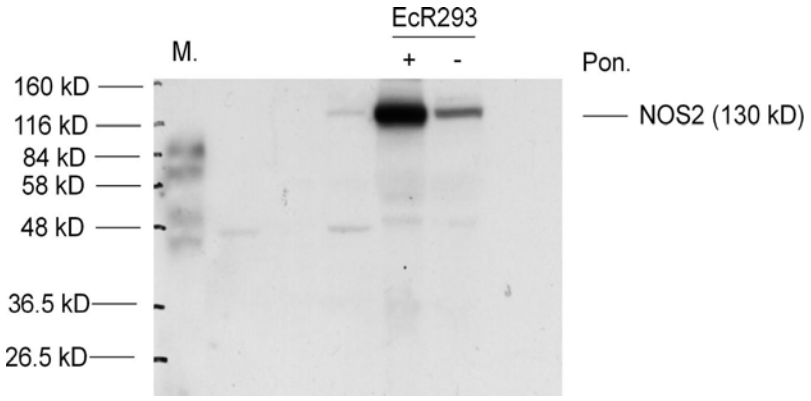


Figure 7: Transient expression of NOS2 in EcR293 cells. The cells were induced (+) or uninduced (-) by ponasterone A for 48 hours. M. =standard molecular protein marker. The mass of NOS2 is about 130 kD.

In this experiment, the first antibody (anti-NOS2 polyclonal rabbit antibody) was diluted at 1:5.000 ratios in I-Block solution, and the secondary antibody was used at 1:10.000. In the Figure 7 the control sample uninduced (-) by ponasterone A showed less expression of NOS2, while the sample induced (+) by 5 μM ponasterone A for 48 hours showed a significant expression of NOS2. From the results of the Western blot, cells induced by ponasterone A expressed NOS2 proteins at the expected molecular mass of about 130 kD. The expression of NOS2 in the control cells (-), which also transfected by pIND-NOS2, but not induced (-) by ponasterone A was less expressed.

The results from immunofluorescent staining and Western blot of transiently transfected cells indicated that pIND-NOS2 construct was inducible by ponasterone A, the protein localized correctly, and the molecular mass corresponded to the known mass of the NOS2 protein.

3.1.3 Characterization of stable clones in EcR293 cells

3.1.3.1 Establishment of stable clones and Western blot analysis

To explore the functions of the biological and pathological influences of NO in human cells, stable NO producing cell lines were developed. The gene construct pIND-NOS2 was transfected into EcR293 cells and clones allowed to grow under antibiotic selection.

EcR293 cells were seeded in the 6-well plates and 5 μg DNA was used to perform transfection. After culturing for 24 h, the cells were trypsinized and 3.000-5.000 cells were seeded per into one 10 cm culture dish. The cells were selected by medium containing 400 μg/ml G418 and 400 μg/ml Zeocin. After approximately 3 weeks of culture under selection conditions, many small colonies had grown. Well isolated colonies were carefully picked by a column cylinder, and transferred into 12-well plates for expansion in selection-medium. When the clones were grown to about 70 % confluency in 12-well plates, and they were trypsinized, transferred into the 6-well plates for further expansion in selection medium. Finally the clones were cultured in 10 cm dishes for freezing, expansion and analysis. The established clones were termed EcR293-NOS2 clones.

The EcR293-NOS2 clones were picked and expanded, the analysis of inducible NOS2-expression in these cells was performed. For this goal, many different stable clones were chosen for analysis.

First the expression of NOS2 was investigated by Western blot with or without ponasterone A induction. Together 61 clones were picked and 47 clones were analyzed by Western blot for the expression of NOS2.

The information about picked clones:

Performance	Numbers (clone cells)	Percentage
Clones picked	61	-
Clones analysed by Western blot	47	77 % (Com. with total picked clones)
NOS2 expression was positive	28 total	59 % (Com. with analysed clones)
	(10 Cons. and 18 Ind.)	
NOS2 expression was negative	19	40 % (Com. with analysed clones)

Table 1 The statistic of analysis for established EcR293-NOS2 clone cells.
Cons.=constitutively; Ind.=inducible; Com.=comparison

The EcR293-NOS2 clone cells were analysed by Western blot with or without ponasterone A induction. Of 61 clones picked, 47 cell clones were analysed by Western blot. The expression of NOS2 were detectable in 28 clones and in 18 EcR293-NOS2 cell clones was inducible NOS2 expression. Nineteen cell clones showed no expression of NOS2.

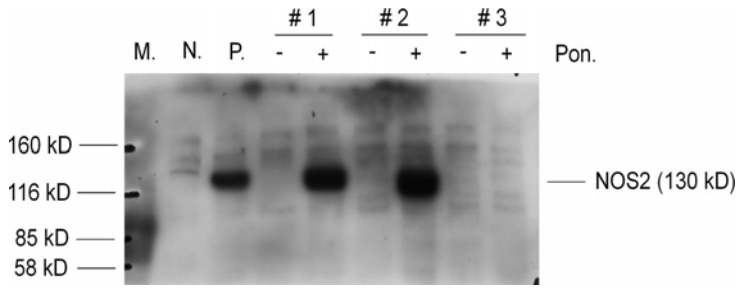


Figure 8: The expression of NOS2 was analysed by Western blot in different cell clones (clone #1, #2 and #3). The cells were induced (+) or uninduced (-) by ponasterone A. M.= standard molecular protein; N.= negative control; P.= positive control of NOS2 expression.

Figure 8 shows NOS2 expression in different EcR293-NOS2 cell clones by Western blot. The expression of NOS2 was detectable in clone #1 and #2, but no expression of NOS2 was detected in EcR293-NOS2 clone #3.

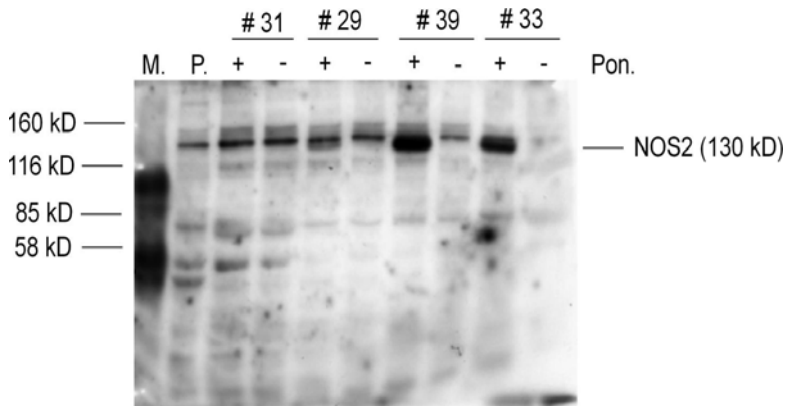


Figure 9: NOS2 expression was analysed by Western blot in different cell clones (clone #31, #29 #39 and #33). The cells were induced (+) or uninduced (-) by ponasterone A. M. = standard molecular protein; P. = positive control of NOS2 expression.

The Western blot results shown in Figure 9 that NOS2 was constitutively expressed in EcR293-NOS2 clone #31 and #29 after the cells were induced (+) or uninduced (-) by ponasterone A. However in clone #33 and #39 the expression of NOS2 was inducible.

The different EcR293-NOS2 clones (clone #45, #53, #33, #35, #15) were chosen for the analysis to test doses dependent induction of NOS2. They were seeded in 6-well

plates and induced by ponasterone A at concentration of 0, 0.5, 1, 3, 10 µM. Finally the cells were harvested in lysis buffer and analysed by Western blot.

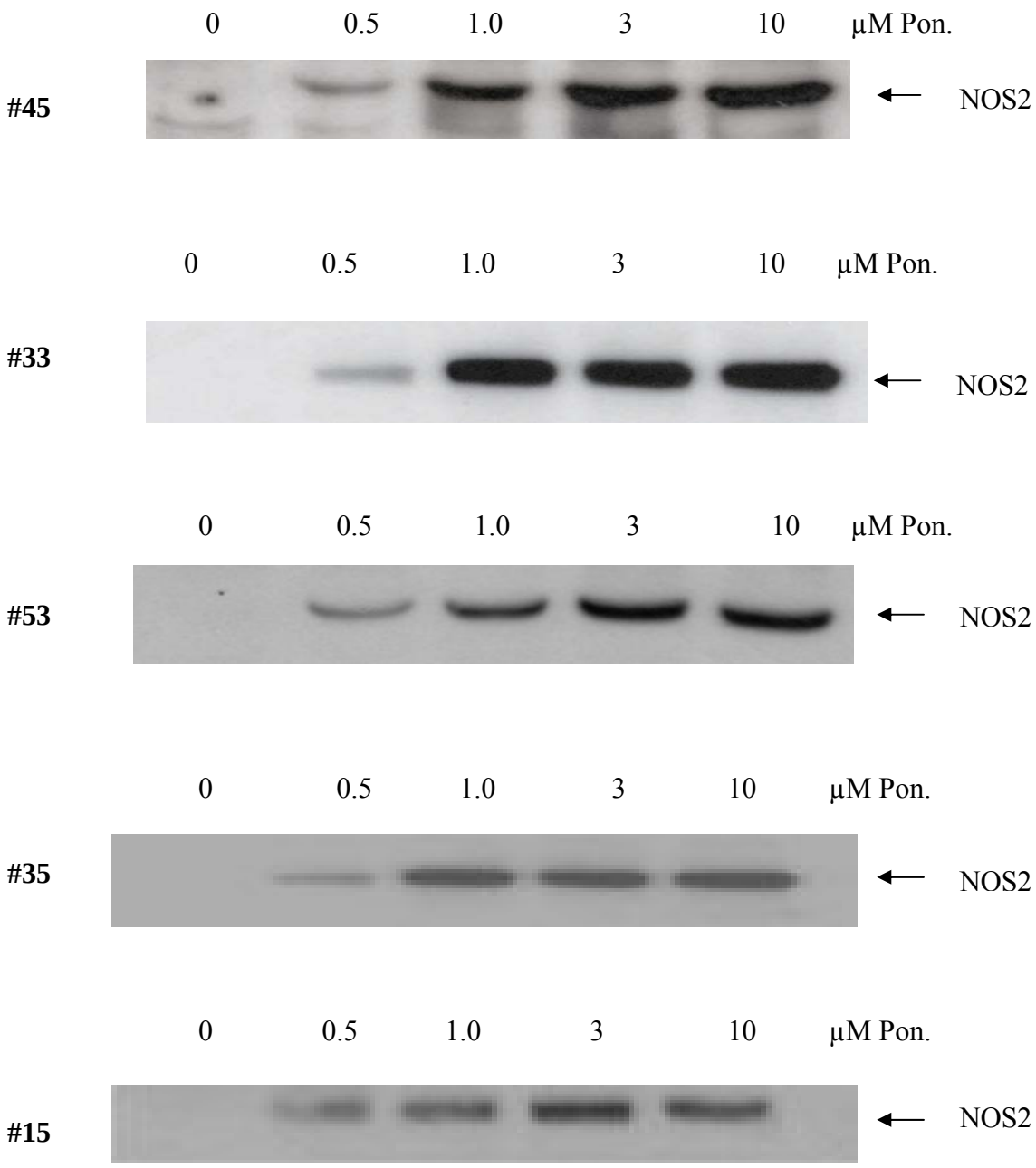


Figure 10: Expression of NOS2 in different EcR293-NOS2 clones. The clones #45, #53, #33, #35, and #15 were used for this experiment. The samples uninduced by ponasterone A (0 µM) showed that the expression of NOS2 was not detectable. Comparing with the control (0 µM Pon.), all the samples induced with ponasterone A (0.5, 1, 3, 10 µM) expressed high level of NOS2.

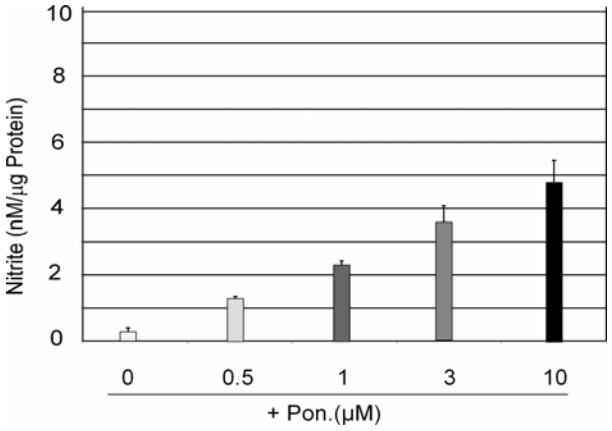
Western blot analysis (Figure 10) shows that there was no NOS2 expression in the control sample (0 μM Pon.) of different EcR293-NOS2 clone cells, but samples induced by different concentrations of ponasterone A show dosis dependent expression of NOS2 in these clone cells. In the EcR293-NOS2 stable clones #45, #33, #53, #25, #15, induction by 0.5, 1, 3, 10 μM ponasterone A resulted in increasing amount of NOS2 expression.

3.1.3.2 Griess assay for NO production

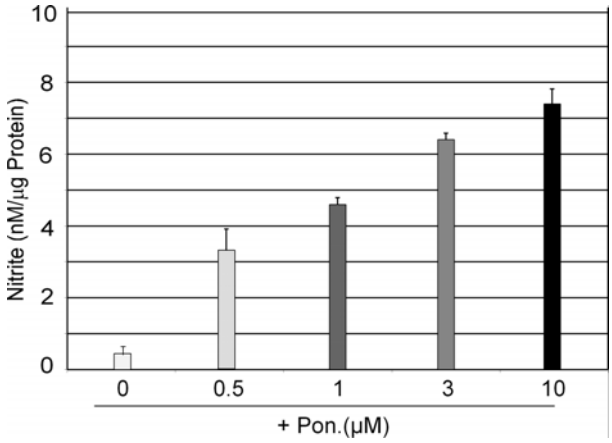
The production of NO from endogenous NOS2 expression in EcR293-NOS2 cells was measured by Griess assay. The Griess assay was used to quantify the biological products (mainly nitrite) from NO. EcR293-NOS2 cell clones were seeded in the 6-well plates, and they were induced by ponasterone A at concentration of 0, 0.5, 1, 3, 10 μM. After 48 h culture, the medium was collected and the cells were trypsinized. Finally Griess assay was performed (methods 2.6.7).

EcR293-NOS2 clones:

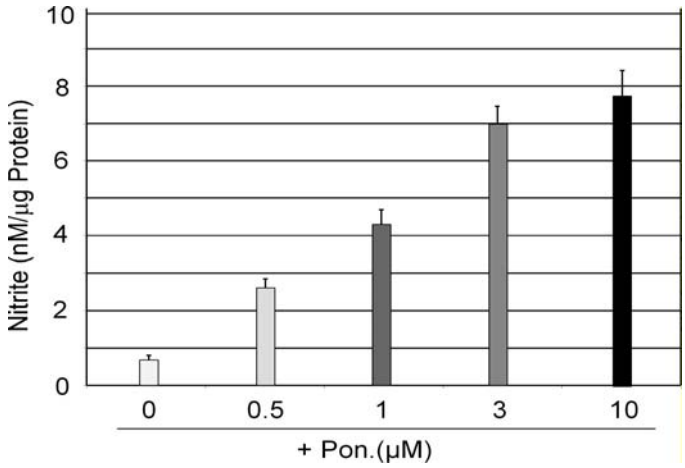
#15



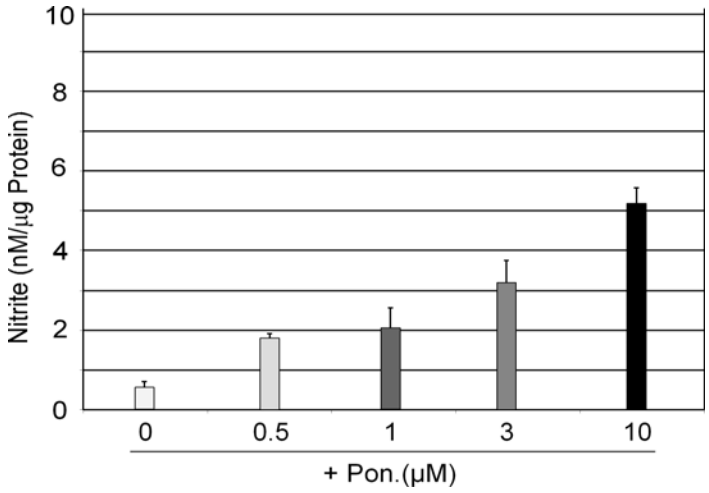
#35



#33



#45



#53

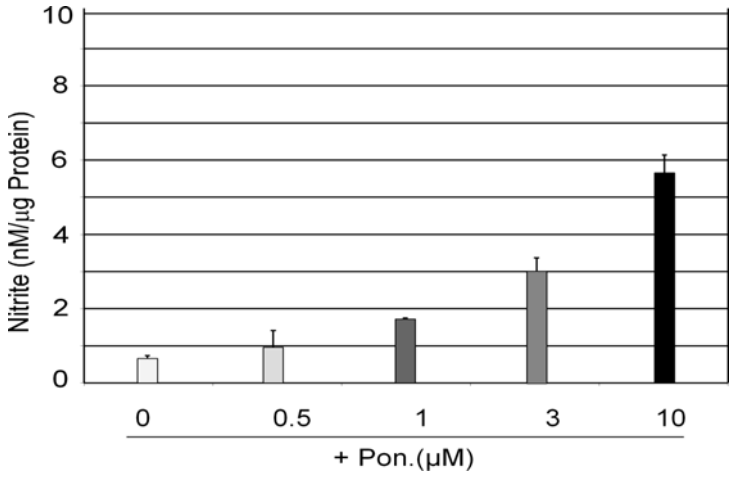


Figure 11: Measurement of nitrite concentration by Griess assay in different EcR293-NOS2 cell clones. The cells were induced by 0.5, 1, 3, 10 µM ponasterone A. The concentration of nitrite is presented at µM/µg protein.

The results of Griess assay (Figure 11) show that the production of NO was induced even at the low concentration of ponasterone A. All stable NO-producing cell clones showed the similar doses dependence; the concentration of nitrite from the expression of NOS2 was correlated with increasing concentration of ponasterone A (from 0.5 μM to 10 μM) in a doses-dependent way. However different EcR293-NOS2 stable cell lines showed differences in the concentration of nitrite. In EcR293-NOS2 #33 and #35 cells, the concentration of NO production was more than 6 nM/μg and clone #53 showed much lower concentration of nitrite production. In this clone even induction by the high concentration of ponasterone A (10 μM ponasterone), the amounts of nitrite production were nearly 2 nm/μg. Together Griess assay, NO production was induced from all of the stable clones (#15, #33, #35, #45, and #53) in a doses-dependent way after induction by ponasterone A.

3.1.3.4 Kinetic of NOS2 expression

A time course for induction of NOS2 after treatment with ponasterone A was performed for exploring when the peak of NOS2 expression in EcR293-NOS2 cells was.

The EcR293-NOS2 #33 cells were seeded in the 6-well plates, and they were induced by ponasterone A for 0, 8, 12, 24, 36, 48, 60 h. Finally the cells were washed by normal PBS one time and harvested with 1 % NP-40 solution. The samples were incubated on ice for 15 min and centrifuged. The protein concentrations of all samples were measured and 40 μg protein was used for Western blot on a 8 % SDS-PAGE gel.

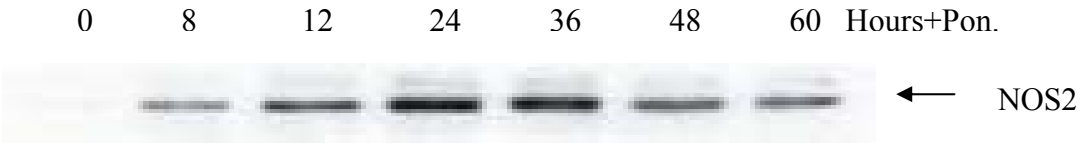


Figure 12: Time kinetic of NOS2 expression in EcR293-NOS2 #33 cells. Clone #33 was induced by 5 μM ponasterone A for 0, 8, 12, 24, 36, 48, 60 h. Western blot analysis indicates that NOS2 expression peaks between 12 to 48 h.

From Western blot analysis (Figure 12) it indicated that the expression of NOS2 was increased to the higher point after the induction for 24 h by ponasterone A, and revealed maximal expression levels between 12 and 48 h, however continuous expression of NOS2 could be detected throughout the time course of the experiment

3.2 Analysis of CD95-, TRAIL- or CTL-mediated apoptosis signal in NOS2-expressing cells

3.2.1 Apoptosis sensitivity

3.2.1.1 CD95-induced apoptosis

CD95-mediated apoptosis sensitivity in EcR293 cells

The apoptosis sensitivity in EcR293 cells was tested for determining the appropriate concentration of anti-APO-1 after cells were treated with anti-APO-1, agonistic CD95 antibody. For this analysis, MTT measurements were performed. EcR293 cells were seeded in the 96-well plates, and the cells were treated with anti-APO-1 at different concentrations (10, 100, 200, 500, and 1.000 ng/ml) in quadruplicate for 24 h. MTT assay was performed according to the method (2.7.6.3) and the data was analysed.

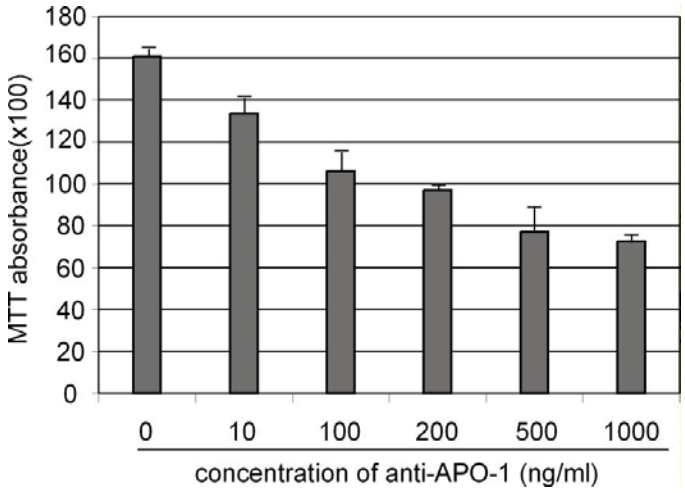


Figure 13: The apoptotic sensitivity was analysed in EcR293 cells after triggering with anti-APO-1 at different concentrations for 24 h.

Figure 13 shows in comparison with the control sample (0 ng/ml), those samples treated with anti-APO-1 (+ anti-APO-1, from 10 to 1.000 ng/ml) were induced to undergo apoptosis. At the concentration of 500 ng/ml and 1000 ng/ml, there were nearly more than 50 % apoptotic cells. This experiment demonstrated that the EcR293 cells are sensitive for CD95-induced apoptotic through stimulation with 200-500 ng/ml CD95-agonistic antibody anti-APO-1.

The apoptotic sensitivity was not influenced by ponasterone A

Another question is, whether the ecdysone analog "ponasterone A" influences the apoptosis sensitivity induced by anti-APO-1. For analyzing the influence of ponasterone A on the apoptosis sensitivity, EcR293 cells were seeded in 6-well plates about 70% confluency, and the cells were treated with different concentration of ponasterone A (from 0, 0.5, 1, 3, 10 µM concentration), after 24 h, the medium was changed (containing the same concentration of ponasterone mentioned above) and treated by 300 ng/ml anti-APO-1 for another 24 h. During these steps, the ponasterone A first mixed with the medium, and afterwards the medium containing ponasterone A was carefully added to the cells to assure accuracy.

Following the treatment with anti-APO-1 for 24 h, the medium containing detached cells were collected, and the attached cells were harvested by centrifugation after trypsination. The cells were resuspended in PBS, fixed by pre-cold 100 % ethanol and finally the cells were stained by propidium iodide (PI). They were incubated at 4 °C overnight and then the samples were measured by FACS.

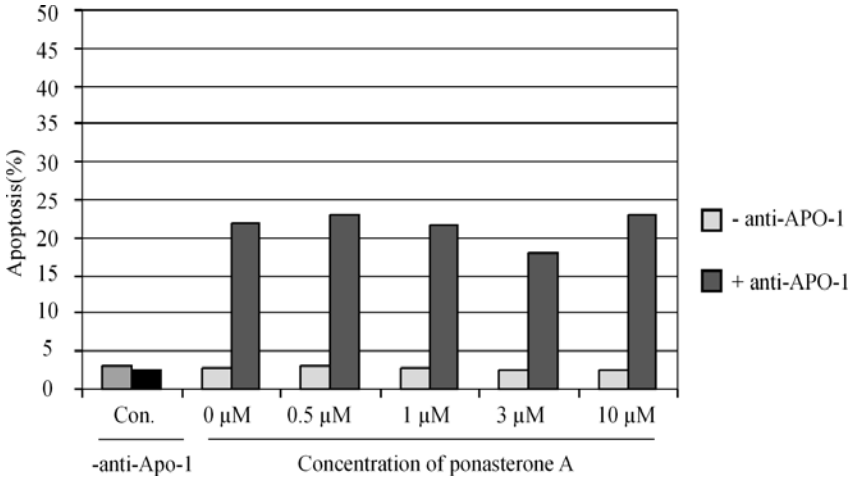


Figure 14: The apoptosis sensitivity was not influenced by ponaterone A in EcR293 cells.

The apoptosis sensitivity induced by anti-APO-1 was between 18 % and 23 %. These results showed that the apoptosis sensitivity of EcR293 cell was not influenced by ponasterone A after anti-APO-1 induction.

Comparison of apoptosis sensitivity between EcR293 and EcR293-NOS2 cells

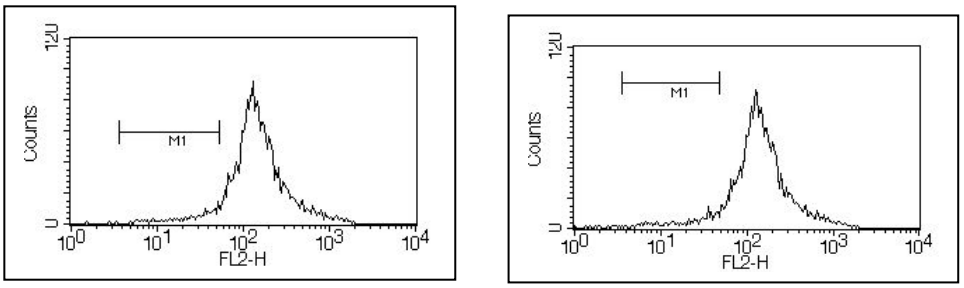
For investigating the sensitivity between NOS2-expressing cells and control cells after inducing CD95-mediated apoptosis, EcR293 and EcR293-NOS2 #33 cell lines were used. The cells were seeded in the 12-well plates, they were induced by ponasterone for 24 h, and afterwards the cells were treated with 300 ng/ml anti-APO-1. After the treatment for 24 h, the cells were harvested, fixed by 100 % cold ethanol and stained with propidium iodide (PI) for FACS analysis.

EcR293:

A.

- anti-APO-1

0 10 µM Pon.

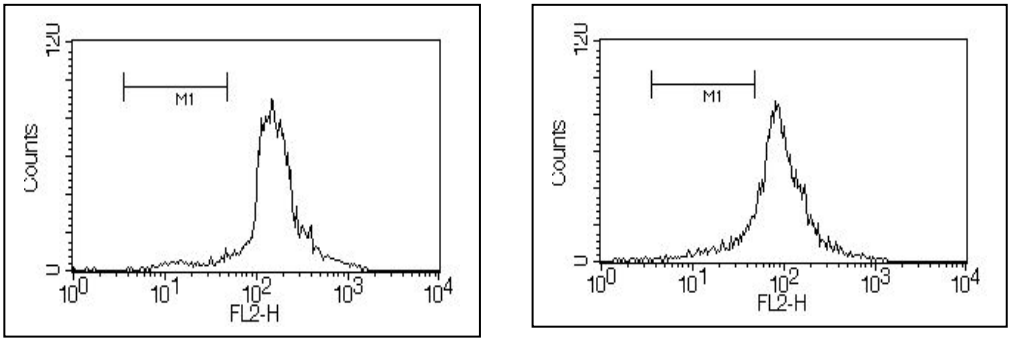


4.0 % 4.96 % Apoptosis

+ anti-APO-1

B.

0 10 µM Pon.



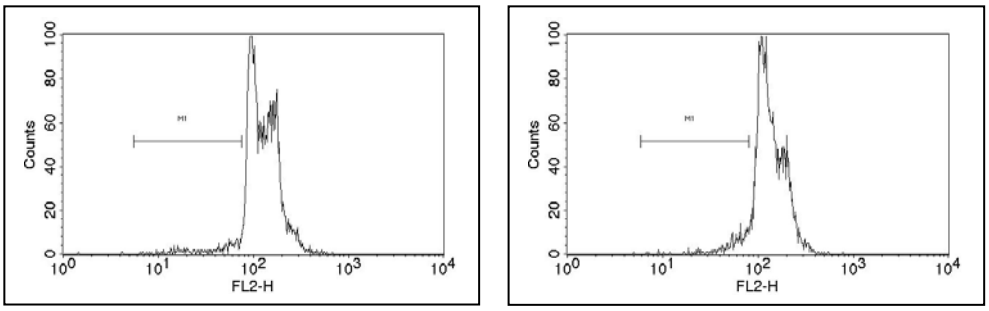
13 % 13.8 % Apoptosis

EcR293-NOS2 #33

C.

- anti-APO-1

0 10 µM Pon.



3.86 % 4.80 % Apoptosis

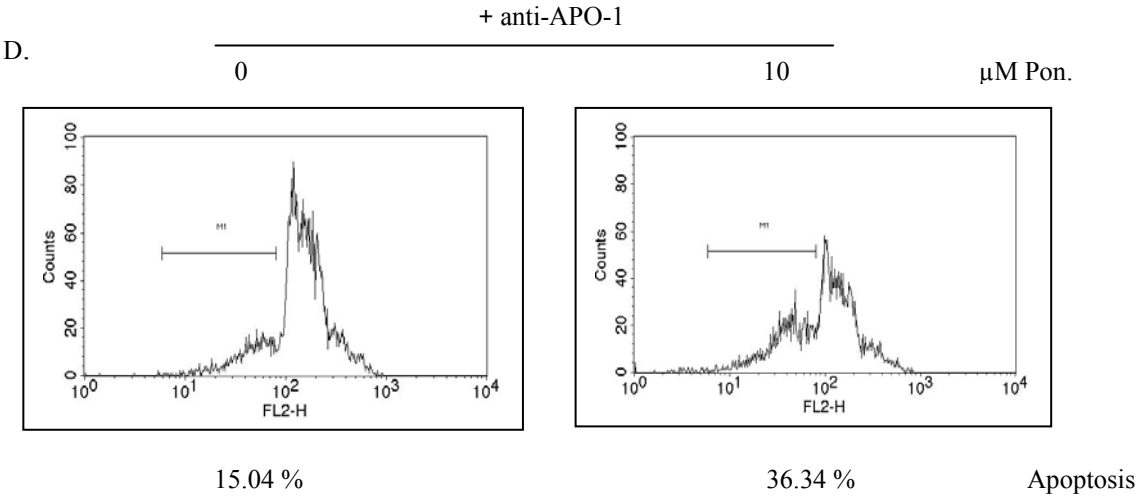


Figure 15: The apoptosis sensitivity in EcR293 and EcR293-NOS2 #33 cell lines analyzed by FACS.
The EcR293 cells were induced by ponasterone (0, 10 µM) for 24 hours, and treated without anti-APO-1 (A), with 300 ng/ml anti-APO-1 (B). The EcR293-NOS2 #33 cells were induced by ponasterone (0, 10 µM) for 24 h, and treated without anti-APO-1 (C), with 300 ng/ml anti-APO-1 (D).The M1 peak shows apoptotic sub-G1 cells. .

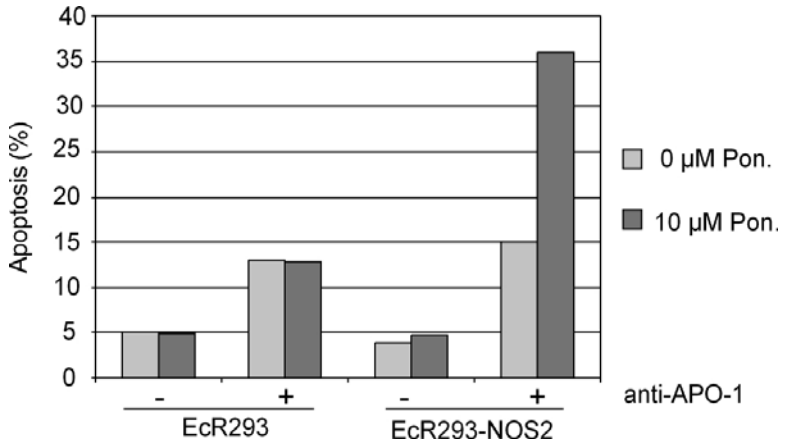


Figure 16: Apoptosis sensitivity analysed by FACS in EcR293 and EcR293-NOS2 cells.

The results from FACS analysis (Figure 15 and 16) show the difference of apoptotic sensitivity between EcR293 and EcR293-NOS2 cells during anti-APO-1 triggered apoptosis. In EcR293 cells, the uninduced (0 µM Pon.) or induced cells (+ 10 µM Pon.) showed nearly same percentage of apoptosis. Comparing with the control cells (-

anti-APO-1), there was about 12 % apoptotic cells after anti-APO-1 treatment in the cells (EcR293 + 0 μM Pon. and EcR293 + 10 μM Pon.). In EcR293-NOS2 cells, the uninduced cells (0 μM Pon.) showed only 15 % apoptotic cells, but cells induced by ponasterone A (+ 10 μM Pon.) showed more than 35 % apoptotic cells.

These results show that endogenously produced NO plays a pro-apoptotic role in CD95-mediated apoptosis pathway and NO enhances the apoptotic sensitivity from 15 % up to more than 35 % in the CD95-triggerd apoptosis pathway.

Analysis of apoptosis sensitivity in EcR293-NOS2 cells during induction by ponasterone A

Analysis of apoptotic sensitivity induced by different dosis of ponasterone A was performed in NOS2-expressing clone cell EcR293-NOS2 #33. The cells was seeded in the 12-well plate, they were induced by ponasterone A at 0, 0.5, 1, 3, 10 μM concentration for 24 h, then they were treated with anti-APO-1 at 300 ng/ml. After 24 h treatment the cells were harvested, fixed by 100 % cold ethanol and stained with propidium iodide (PI) for FACS analysis.

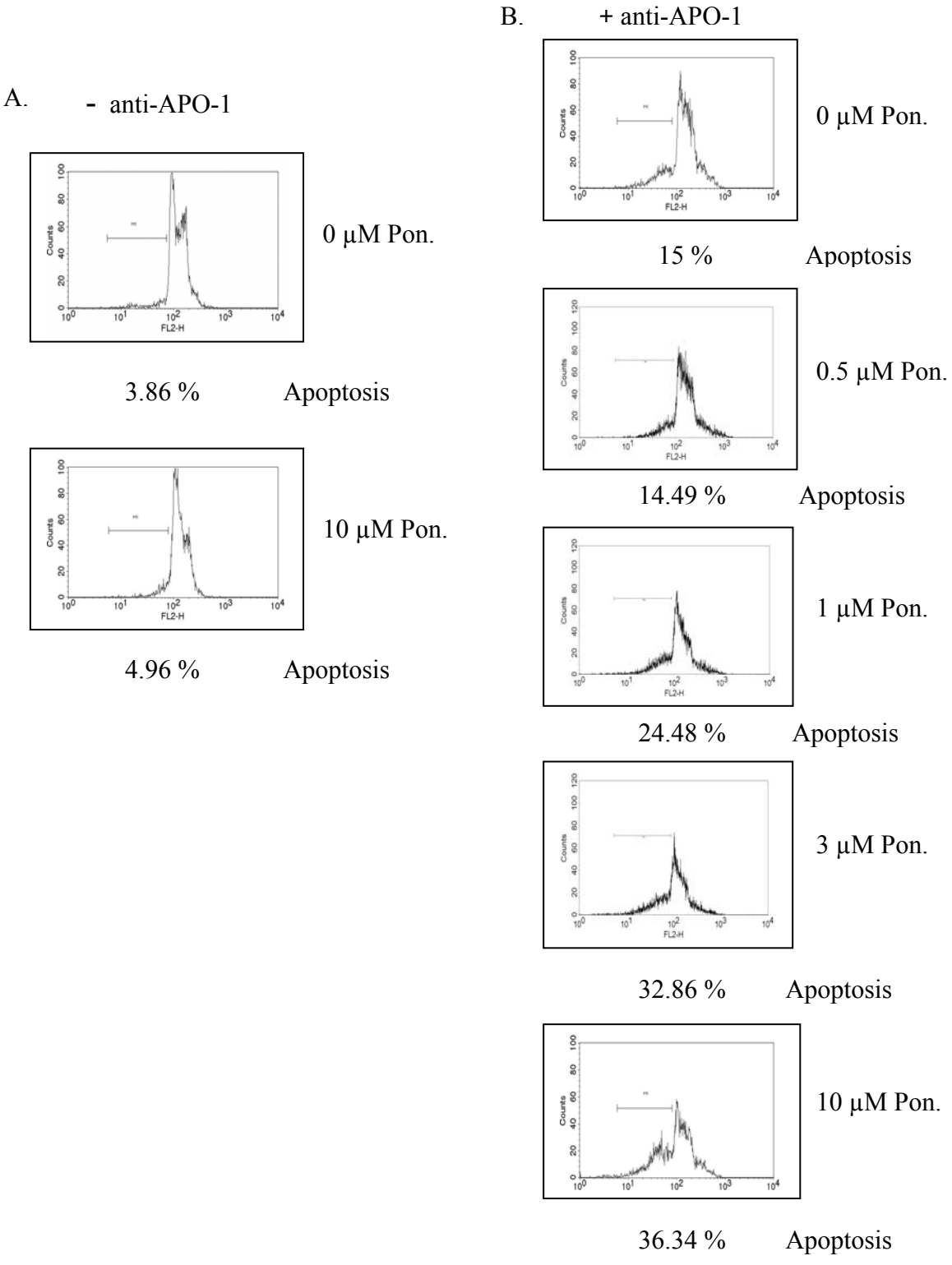


Figure 17: NO increases apoptosis sensitivity induced by anti-APO-1 in EcR293-NOS2 cell #33.
The samples in panel A were induced only by ponasterone A, and in panel B the samples were induced by ponasterone A and treated with anti-APO-1 (300 ng/ml).

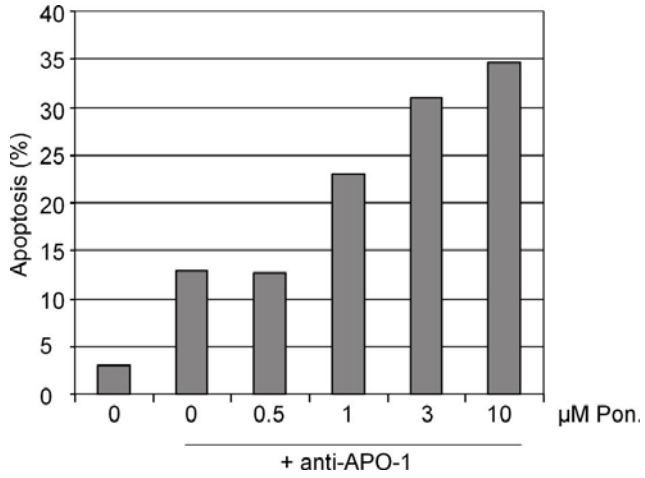


Figure 18: Apoptosis sensitivity was analysed by FACS in EcR293-NOS2 cell #33.
The cells were induced by ponasterone A at the concentration of 0, 0.5, 1, 3, and 10 µM. Apoptotic sub-G1 cells were quantified by FACS analysis.

The FACS analysis in Figures 17 and 18 show that the increased apoptotic sensitivity was induced in a dosis dependent way, when the death signal pathway was triggered by anti-APO-1. Apoptosis was induced by addition of 300 ng/ml anti-APO-1 for 24 h to the cultures. Apoptotic sub-G_1-peak indicates increasing apoptosis sensitivity in NOS2 expressing EcR293-NOS2 #33 cells. Cells induced by 1, 3, 10 µM ponasterone A demonstrated more apoptosis sensitivity compared to the ponasterone uninduced cells (-), the uninduced cells had 15 % apoptotic percentage, but cells induced by ponasterone A (+) showed 24.8 % (1 µM Pon.), 32.8 % (3 µM Pon.) and 35 % (10 µM Pon.) apoptotic percentage. These analyses demonstrated that as the dosis of ponasterone A increased, apoptosis induced by treatment with a constant concentration of the agonistic CD95 antibody, anti-APO-1, proportionally increased compared to control.

Analysis of apoptosis sensitivity in different EcR293-NOS2 cells

For exploring the influence of NO on the apoptotic sensitivity triggered by anti-APO-1 in different EcR293-NOS2 clone cells, EcR293-NOS2 clones #15, #35, #45, #53 were seeded respectively in the 12-well plates, and they were induced by ponasterone A at the concentration of 0, 3, 10 µM for 24 h, then cells were added with 300 ng/ml anti-

APO-1 for another 24 h. Ultimately they were harvested, fixed and stained with propidium iodide (PI) for FACS analysis.

EcR293-NOS2 clones:

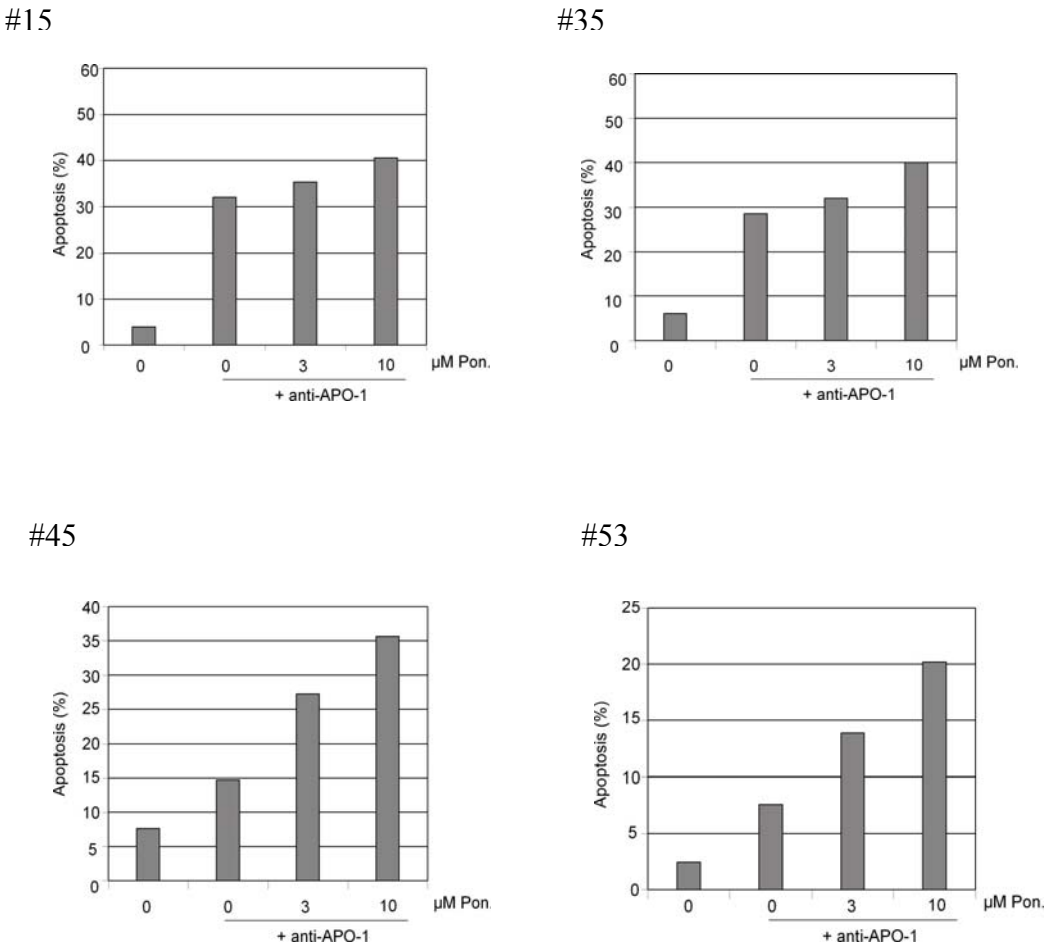


Figure 19: NO enhanced apoptotic sensitivity was analysed by FACS in different EcR293-NOS2 clone cells (#15, #35, #45 and #53).
The cells were induced by 0, 3, 10 µM ponasterone A and treated with 300 ng/ml anti-APO-1, and all of the samples from different clones were harvest at the same time, apoptotic sub-G1 cells were analysed by FACS.

The data in Figure 19 show that the apoptotic effects observed in four independently derived EcR293-NOS2 cell clones (#15, #35, #45 and #53) were nearly similar, namely the increasing apoptosis sensitivity was correlated with a dosis augmented concentration of ponasterone A. These results from Figure 17, 18 and 19 indicated that induced NOS2 expression was responsible for an increased CD95 apoptosis sensitivity in EcR293-NOS2 cells.

3.2.1.2 TRAIL-induced apoptosis

To investigate the influence of NO on the TRAIL-induced apoptosis sensitivity, EcR293-NOS2 #33 cells were seeded in the 6-well plates and induced by ponasterone A for 24 h. Then the cells were untreated (-) or treated (+) with 50 ng/ml TRAIL for another 24 h. Finally they were harvested and centrifuged, fixed by 100% cold ethanol and stained by propidium iodide (PI) and measured by FACS.

.

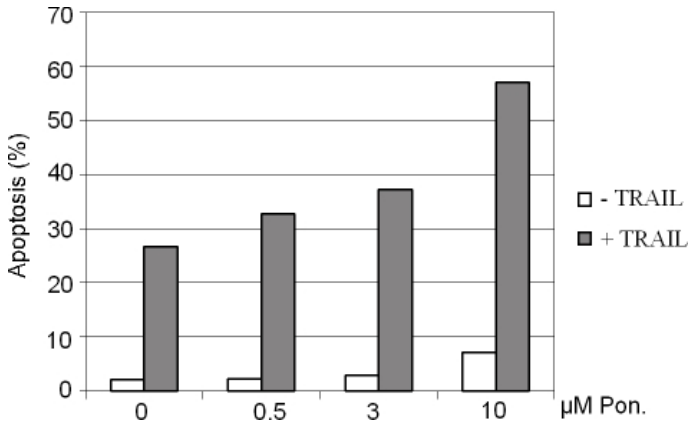


Figure 20: TRAIL-mediated apoptosis sensitivity was analysed in EcR293-NOS2 #33. The cells were seeded in 400 thousands cells/well in the plates, induced by ponasterone A. of 0, 0.5, 3 and 10 µM concentration. Apoptotic sub-G1 cells were analysed by FACS.

The FACS analysis (Figure 20) shows that comparing to the untreated samples (- TRAIL), the treated samples (+ TRAIL) showed at least more than 25% apoptotic cells. The increasing apoptotic sensitivity induced by TRAIL was proportionally correlated with the increasing concentration of ponasterone A (0.5, 3 and 10 µM) in EcR293-NOS2 cells #33, these results indicate that the apoptosis induced by TRAIL was enhanced by the expression of NOS2.

3.2.1.3 CTL-induced apoptosis

The CD95 receptor/ligand-system mediates perforin-independent cytotoxic T-cell killing of tumor cells. To explore whether cytolytic effector functions of CTLs are influenced by NO produced from NOS2 expression, cocultured experiments were

performed with antigen-specific HLA-A*0201 restricted cytotoxic T lymphocytes (CD8xA2k^b Flu and CD8 allo A2 were used) and EcR293-NOS2 cells as targets.

CTL: CD8xA2k^b Flu

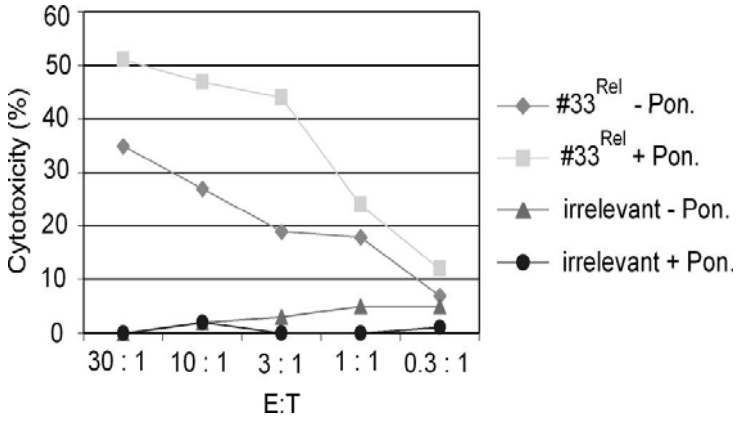


Figure 21: Cytotoxic action of CTL is significantly increased in NOS2 expressing cells.
Coculture experiment were performed with allogenic T cells (CTL CD8xA2k^b Flu) and EcR293-NOS2 #33 target cells. NOS2 expression was induced with 5 μM (+ Pon) or without (- Pon) ponasterone A at the indicated effector to target ratios. An irrelevant peptide were plused in target cells as the control.

CTL: CD8 allo A2:

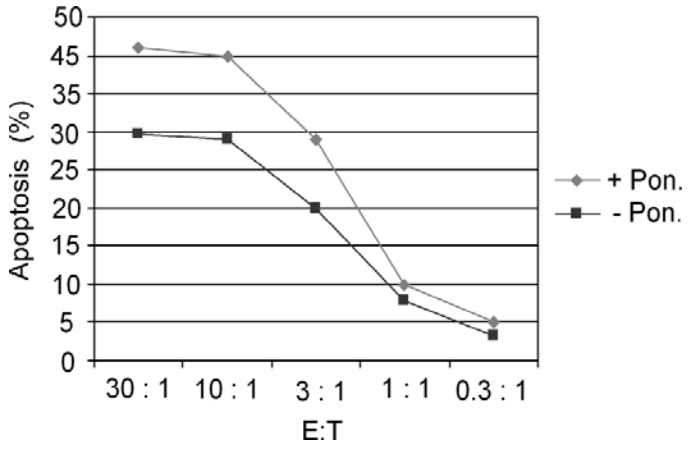


Figure 22: CTL-mediated apoptosis is significantly increased in NOS2 expressing cells.
Coculture experiment were performed with allogenic T cells (CTL CD8 allo A2) and EcR293-NOS2 #33 target cells. NOS2 expression was induced with 5 μM (+ Pon) or without (- Pon) ponasterone A at the indicated effector to target ratios.

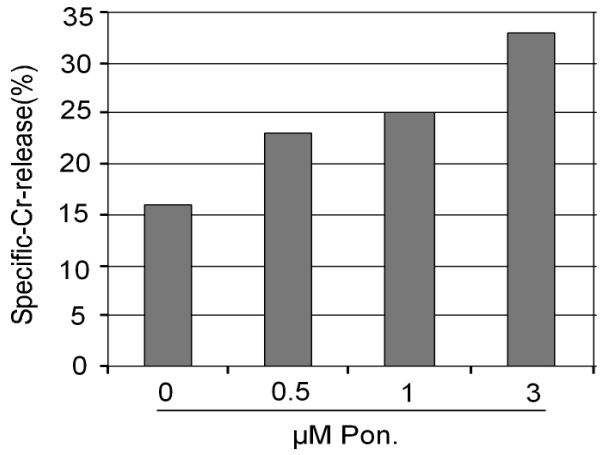


Figure 23: Dose-dependent induction of NOS2 in EcR293-NOS2 #33 cells correlates with apoptosis sensitivity induced by cytotoxic T cells (CTL CD8 allo A2)

The results from co-culture experiments in Figure 21 show that a substantial increase in the cytotoxic action of T cells in EcR293-NOS2 targets was observed when NOS2 was expressed by induction with ponasterone A. The NO producing target cells pulsed with an irrelevant peptide were not lysed during the time course of the co-culture experiments excluding the possibility of autocrine suicide (Figure 21). This effect was dependent on the dosis of ponasterone A (Figure 23) indicating that increasing NO levels in targets correspondingly increased their susceptibility for the killing by CTLs (Figure 22). Taken together the results in Figure 21, 22 and 23 demonstrate that NO increases the sensitivity of EcR293-NOS2 cells to apoptosis induced the cytolytic action of CTLs.

3.2.2 Apoptosis signalling pathway

3.2.2.1 Caspases activities

Activation of caspase -8, -9 and -3 in CD95-mediated apoptosis pathway

For investigating the effect of NOS2 induction on caspase activity during CD95-mediated apoptosis, EcR293 and EcR293-NOS2 cells were seeded in 6-well plates, induced by ponasterone A and treated with anti-APO-1. Afterwards the cells were

harvested for performing caspase assay. 10 µg protein was used for caspase-3 activity analysis, and 30 µg protein were used for analysis of caspase-8 and caspase-9 activities. Samples and the responsible caspase substrates for assay were added to the reaction buffer containing 1 X DTT. The samples were incubated at 37 °C for 2 h. Finally the fluorescent intensity was measured on fluorescent ELISA-reader.

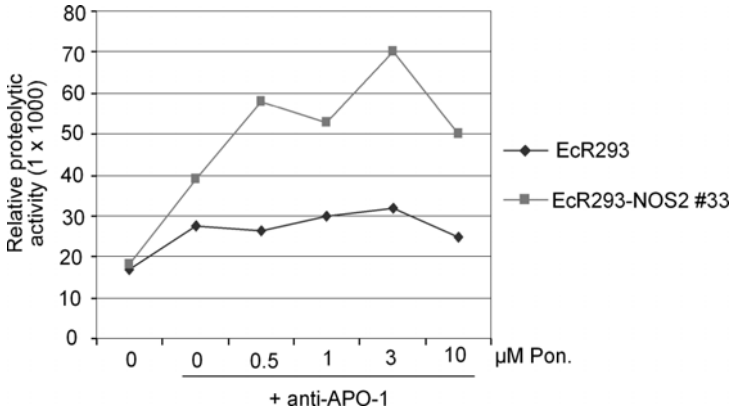


Figure 24: Caspase-8 activity during CD95-induced apoptosis was analysed in EcR293 and EcR293-NOS2 # 33 cells.
The cells were induced by ponasterone A in different concentrations (0, 0.5, 1, 3, 10 µM) for 24 h. The caspase was activated by treatment with 300 ng/ml anti-APO-1.

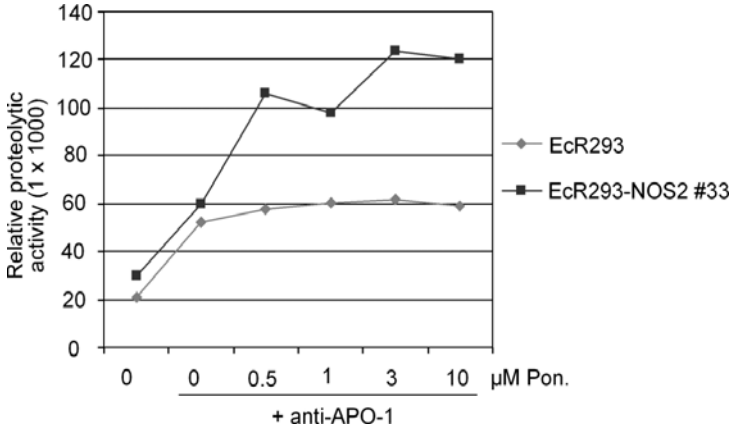


Figure 25: Caspase-9 activity during CD95-induced apoptosis was analysed in EcR293 and EcR293-NOS2 # 33 cells.
The cells were induced by ponasterone A in different concentrations (0, 0.5, 1, 3, 10 µM) for 24 h. The caspase was activated by treatment with 300 ng/ml anti-APO-1.

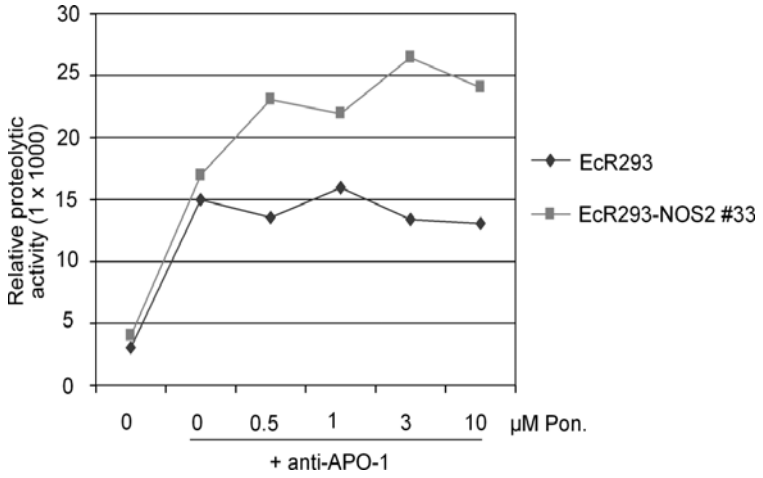


Figure 26: Caspase-3 activity during CD95-induced apoptosis was analysed in EcR293 and EcR293-NOS2 # 33 cells.
The cells were induced by ponasterone A in different concentrations (0, 0.5, 1, 3, 10 µM) for 24 h. The caspase was activated by treatment with 300 ng/ml anti-APO-1.

Figure 24, 25 and 26 show the results that caspases-3, -8 and -9 activations after CD95-stimmulation were enhanced when NOS2 expression was induced in EcR293-NOS2 cells. On contrast the significant increase of caspases activation (caspase-3, -8, and -9) was not observed in EcR293 cells, when cells were induced by different concentration of ponasterone A (0, 0.5, 1, 3 and 10 µM) and in these cells NOS2 expression can not be induced. These results indicate that NO can increase caspase activation during the CD95-mediated apoptosis in EcR293-NOS2 cells.

Activation of caspase -8, -9, -6 and -3 in TRAIL-induced apoptosis pathway

Similar to the CD95-mediated apoptosis pathway, caspase-8 also plays as an „initiator“ of apoptosis in TRAIL-mediated apoptosis pathway, and other caspases, such as caspase-9, -7, -4, -6 and -3 act as „effector“ roles in caspase cascade. To investigate the effect of NOS2 induction on caspase activity during TRAIL-mediated apoptosis, EcR293-NOS2 cells were seeded in the 6-well plates, induced with (+ Pon.) or without (- Pon.) ponasterone A, then treated with 50 ng/ml TRAIL. Afterwards the cells were harvested for caspase assay performance. 10 µg protein was used for caspase-3 activity analysis, and 30 µg protein was used for analysis of caspase-6, -8 and caspase-9 activities. While the proteins were pipetted and the respective caspase substrates for assay were added, and the reaction buffer containing 1 X DTT were pipetted. The samples were incubated at 37 °C for 2 h. Finally the fluorescent intensity was

measured using SAFIRE Doppel-Monochromater. The data were saved and analyzed using Excel software.

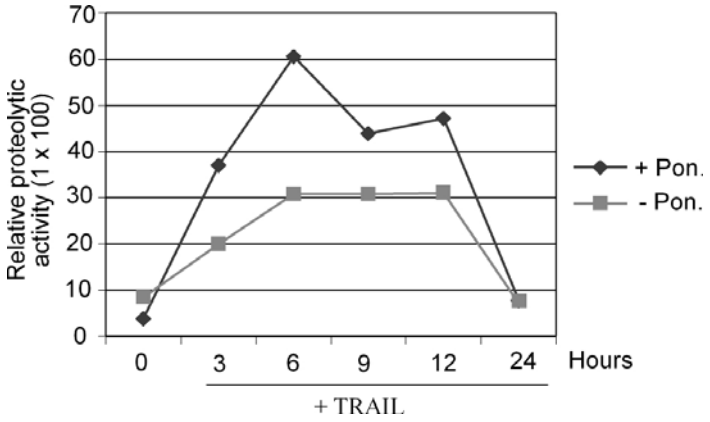


Figure 27: Caspase-8 activity during TRAIL-induced apoptosis was analysed in EcR293-NOS2 # 33 cells.
The cells were induced by ponasterone A (+ Pon) at 3 µM or uninduced (- Pon) for 24 h, then treated with 50 ng/ml TRAIL for 0, 3, 6, 9, 12, 24 h.

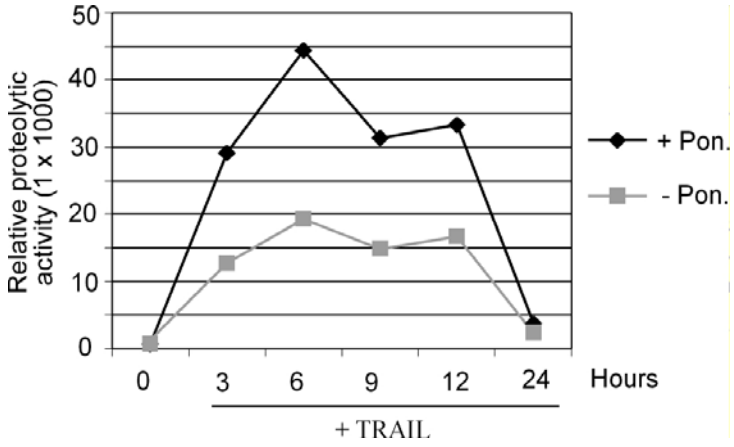


Figure 28: Caspase-3 activity during TRAIL-induced apoptosis was analysed in EcR293-NOS2 # 33 cells.
The cells were induced by ponasterone A (+ Pon) at 3 µM or uninduced (- Pon) for 24 h, then treated with 50 ng/ml TRAIL for 0, 3, 6, 9, 12, 24 h.

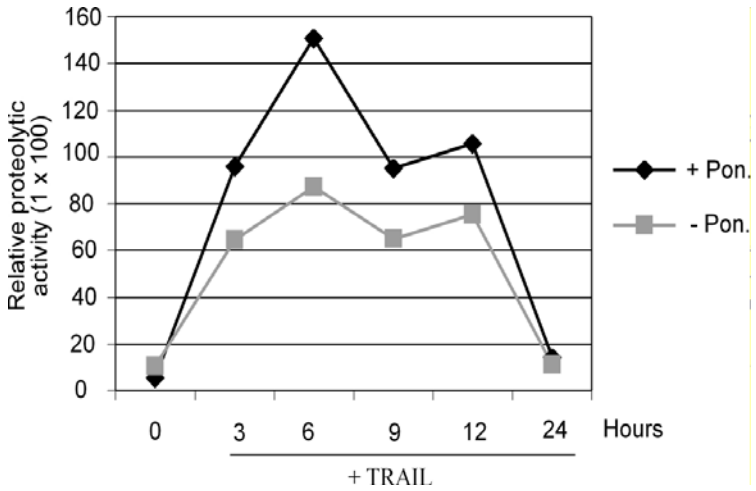


Figure 29: Caspase-9 activity during TRAIL-induced apoptosis was analysed in EcR293-NOS2 # 33 cells.
The cells were induced by ponasterone A (+ Pon) at 3 µM or uninduced (- Pon) for 24 h, then treated with 50 ng/ml TRAIL for 0, 3, 6, 9, 12, 24 h.

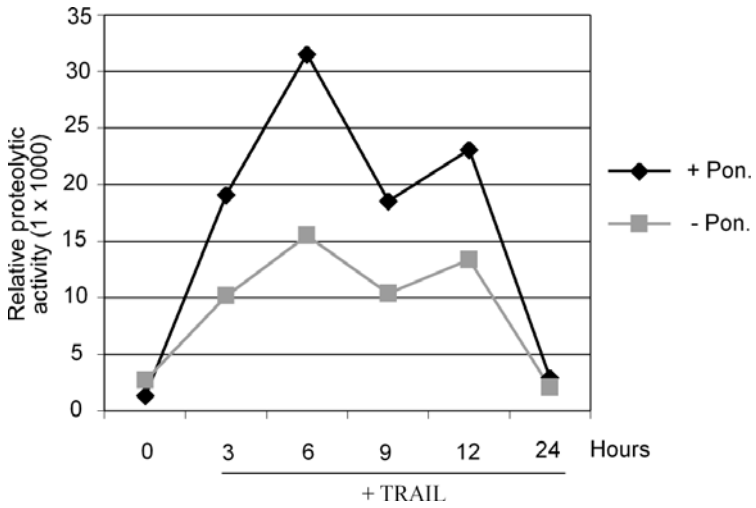


Figure 30: Caspase-6 activity during TRAIL-induced apoptosis was analysed in EcR293-NOS2 # 33 cells.
The cells were induced by ponasterone A (+ Pon) at 3 µM or uninduced (- Pon) for 24 h, then treated with 50 ng/ml TRAIL for 0, 3, 6, 9, 12, 24 h.

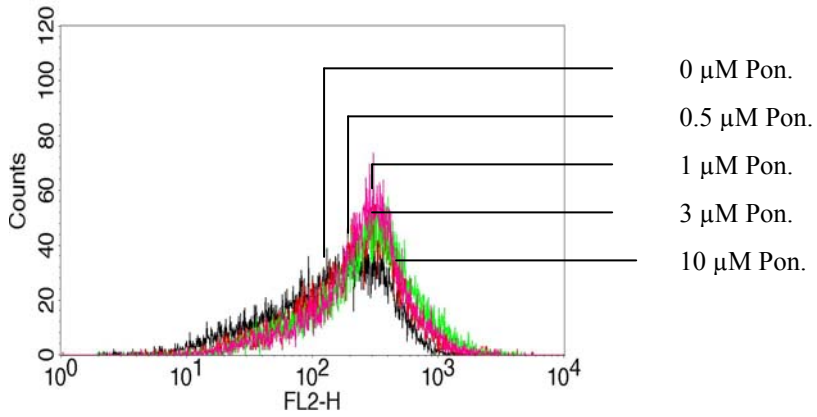
The results in Figure 27, 28, 29 and 30 show that the activation of caspases was increased by the NOS2 expression induced by ponasterone A (+ Pon.). Comparing with uninduced cells (- Pon.), the cells induced to NOS2 expression by ponasterone A from the time course of 3 to 12 h indicated a significantly increasing activation of the

caspase -8, -9, -6 and -3 during the TRAIL-induced apoptosis. The effect of NO on caspase activation was parallel with NOS2 expression.

3.2.2.2 Mitochondrial membrane potential

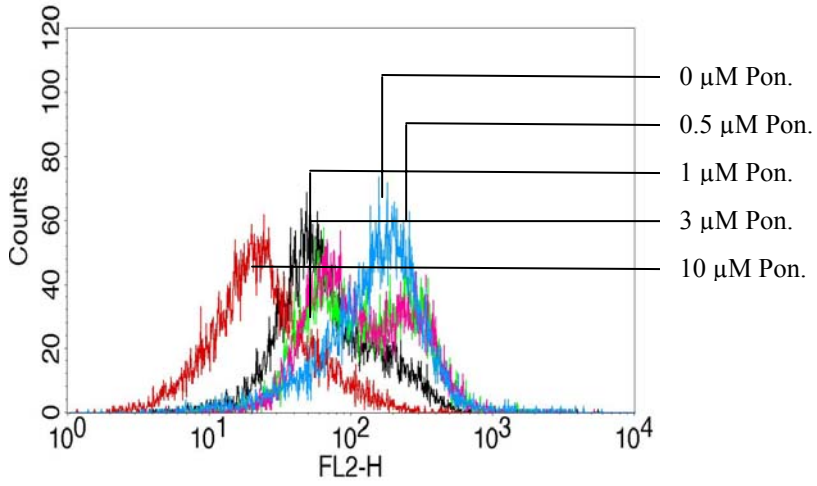
JC-1 staining for the change of mitochondrial membrane potential in CD95-mediate apoptosis pathway

Mitochondria act as a central integrator of the apoptotic response. The loss of mitochondrial membrane potential during CD95-mediated apoptosis pathway was analysed by JC-1 staining in EcR293-NOS2 cells. The cells were seeded in the 6-well plate at about 70 % confluency, induced by ponasterone A and treated with 300 ng/ml anti-APO-1 for 24 h. The cells were harvested for JC-1 staining and measured by FACS.



- anti-APO-1

Figure 31: The change of mitochondrial membrane potential in EcR293-NOS2 #33 cells was analysed without anti-APO-1 stimulation.
NOS2 expression was induced by treatment with 0, 0.5, 1, 3, 10 µM ponasterone A for 24 h, and without anti-APO-1 treatment. JC-1 staining shows rarely loss of mitochondrial membrane potential.



+ anti-APO-1

Figure 32: Loss of mitochondrial membrane potential in EcR293-NOS2 #33 cells after anti-APO-1 stimulation.
NOS2 expression was induced by treatment with 0, 0.5, 1, 3, 10 µM ponasterone A for 24 h, then 300 ng/ml anti-APO-1 was added to induce apoptosis for an additional 24 h. JC-1 staining shows increased loss of mitochondrial membrane potential dependent on NOS2 expression.

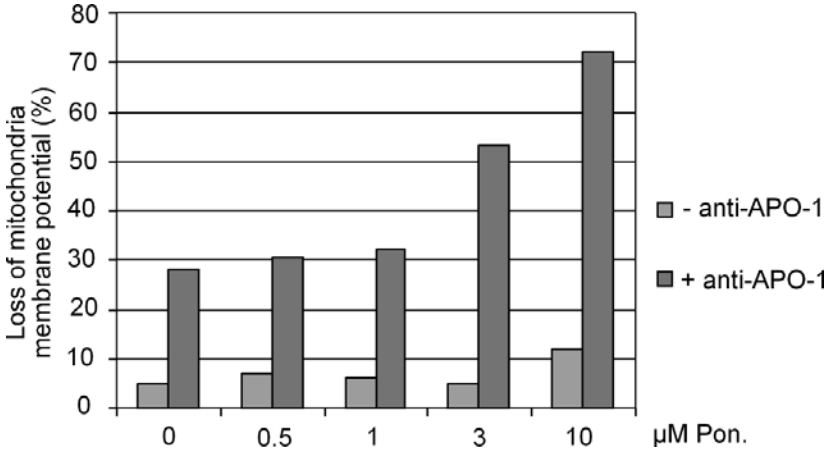


Figure 33: The change of mitochondrial membrane potential was analysed by JC-1 staining.

Figure 31, 32 and 33 show the loss of mitochondrial membrane potential after expression of NOS2 and activation of the CD95 apoptotic pathway in EcR293-NOS2 cells. Quantitative analysis by flow cytometry revealed that short term NO induction sensitized cells to mitochondria membrane potential loss during CD95 apoptosis in a dose dependent manner.

JC-1 staining for the change of mitochondrial membrane potential in TRAIL-induced apoptosis signal

For exploring the change of mitochondrial membrane potential during TRAIL-mediated apoptosis, EcR293-NOS2 cells were seeded in the 6-well plates about 70 % confluency, induced by ponasterone A at concentration of 0, 0.5, 1 and 3 µM and treated with 50 ng/ml TRAIL for 24 h. The cells were harvested, stained with JC-1 and measured on FACS.

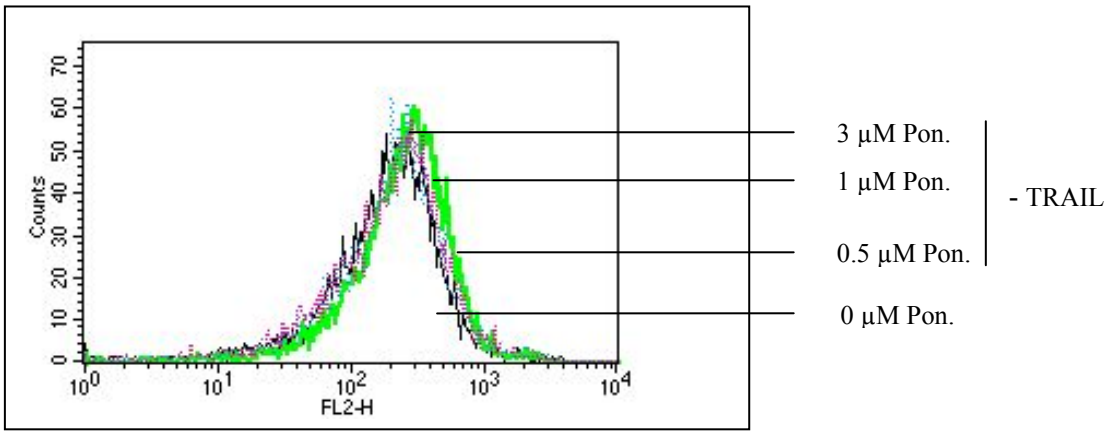


Figure 34: The change of mitochondrial membrane potential in EcR293-NOS2 #33 cells was analysed without TRAIL stimulation (- TRAIL). NOS2 expression was induced by treatment with 0, 0.5, 1, 3 µM ponasterone A for 24 h.

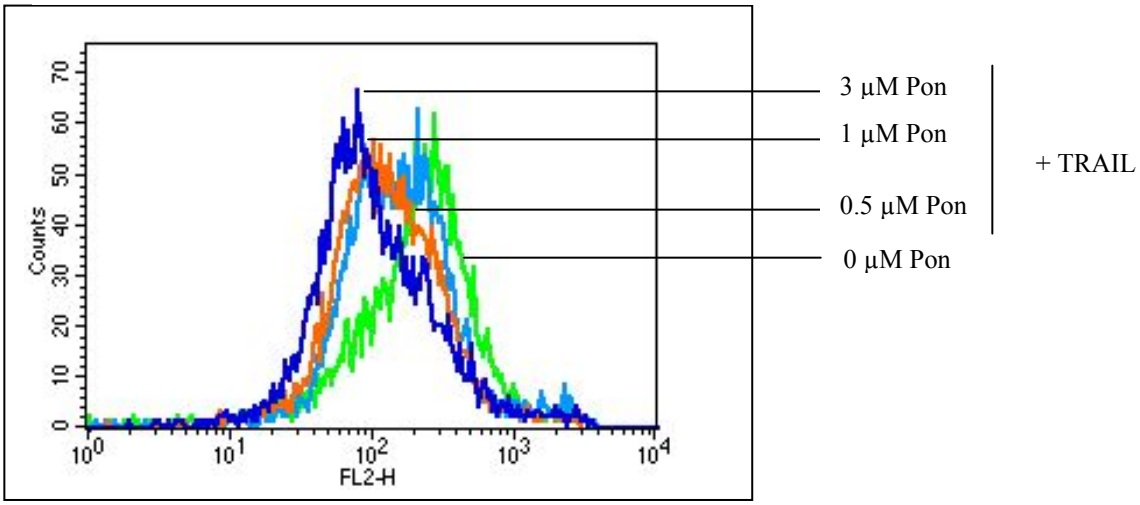


Figure 35: Loss of mitochondrial membrane potential in EcR293-NOS2 #33 cells after TRAIL stimulation.
NOS2 expression was induced by treatment with 0, 0.5, 1, 3 µM ponasterone A for 24 hours, then 50 ng/ml TRAIL was added to induce apoptosis for an additional 24 h.

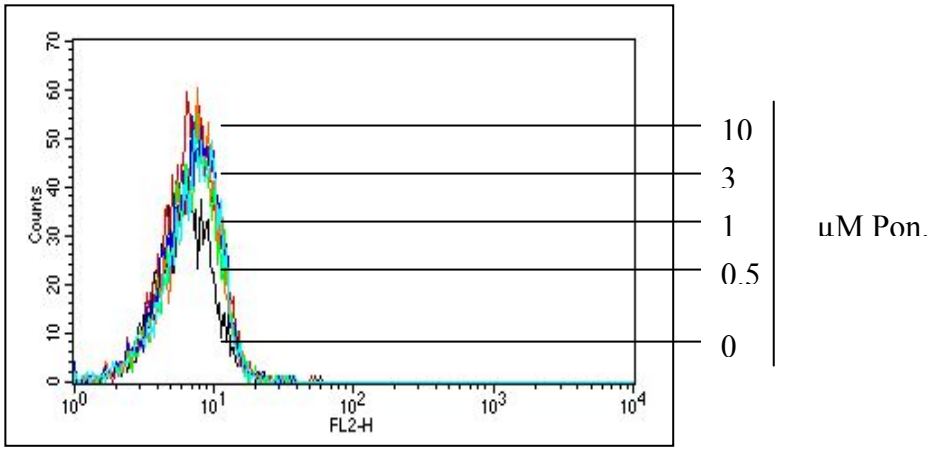
Figure 34 and 35 show that comparing the control cells (- TRAIL), the loss of mitochondrial membrane potential was increased by the expression of NOS2 in the TRAIL-induced EcR293-NOS2 cells. Furthermore, this change of mitochondrial membrane potential were correlated with increasing expression of NOS2 induced by ponasterone A, while the increasing NOS2 expression was proportionally related with the increasing concentration of ponasterone A.

3.3 The expression of CD95- or TRAIL- receptor

3.3.1 The expression of CD95 receptor was analysed by FACS

Based on the effects of NO on CD95- and TRAIL-mediated apoptosis signal pathway, it could be the possible reason that the expression of CD95 or TRAIL receptor was increasingly induced by NOS2 expression. To test this supposition, the expression of CD95 or TRAIL receptor was analysed by FACS. EcR293-NOS2 cells were seeded in the 12-well plates, induced by ponasterone A at concentration of 3 µM. Finally the cells were harvested and stained for analysis on FACS, mouse IgG3 isotype was used as control.

Isotype:



CD95 expression:

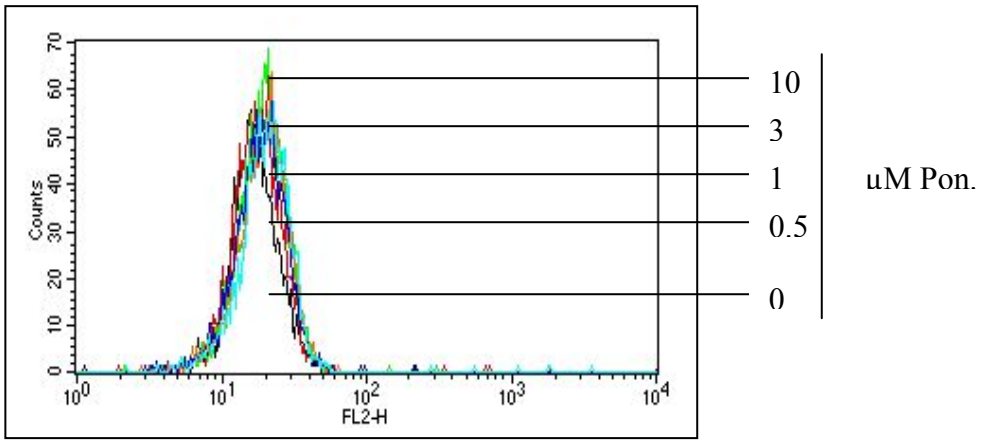


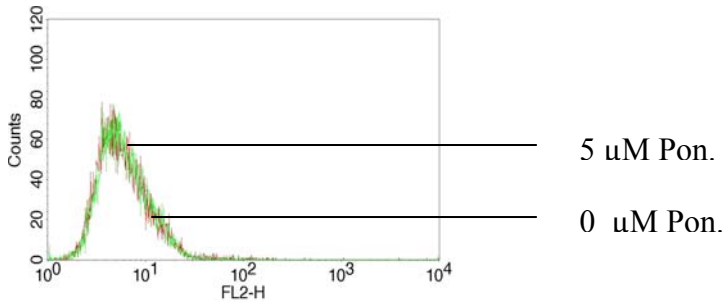
Figure 36: The expression of CD95 receptor on the cell membrane was analysed in EcR293-NOS2 cells.
The cells were induced by 0, 0.5, 1, 3, 10 µM ponasterone A for 48 hr and harvest for analysis on FACS.

Figure 36 shows that the expression of CD95 receptor was not influenced after NOS2 expression induced by increasing concentrations of ponasterone A in EcR293-NOS2 cells.

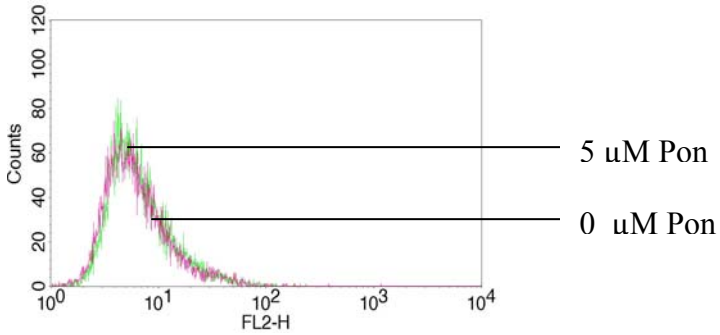
3.3.2 Expression of TRAIL-receptor was analysed by FACS

The expression of TRAIL receptor, specifically TRAIL-R1 and TRAIL-R2, were investigated in EcR293-NOS2 cells. The cells were seeded in 6-well plates, induced by 0 or 5 µM concentration of ponasterone A for 48 h, and then they were harvested and stained for FACS analysis, mouse IgG3 isotype was used as control.

Isotype:



Expression of TRAIL-R1:



Expression of TRAIL-R2:

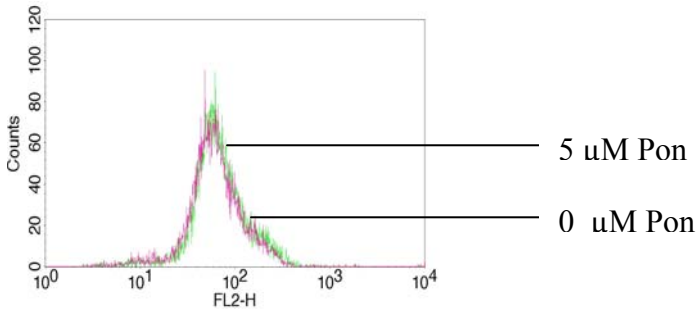


Figure 37: The expression of TRAIL receptors (TRAIL-R1 and TRAIL-R2) were analysed by FACS.
NOS2 expression was induced by 0 and 5 μM ponasterone A for 48 h, stained with anti-TRAIL-R1 or anti-TRAIL-R2 antibody and analysed by FACS.

The results of FACS analysis (Figure 37) reveal that the expression of TRAIL-receptors (mainly TRAIL-R1 and TRAIL-R2) were not influenced by the NOS2 expression induced by 5 μM ponasterone A comparing with the uninduced cells (0μM Pon.) in EcR293-NOS2 cells.

Together the results of CD95- or TRAIL-receptor expression show that the expression of both receptors are not changed by NOS2 expression induced by ponasterone A in cells. Thus, the increased sensitivity to CD95- or TRAIL-mediated apoptosis is not due to an increase in receptor expression.

3.4 Influence of long term NO exposure

3.4.1 Cell growth

Further analysis of the NO effect on cells, the influence of NO on cell growth or apoptosis sensitivity without any stimuli was investigated. Growth properties under uninduced (- Pon.) or induced (+ Pon.) conditions were analysed in EcR293 and EcR293-NOS2 cells. The cells were seeded in 96-well plates and induced as indicated condition in the Figure. Cell-Titer Glo viability assay was used for measuring the change in viable cells.

A. EcR293

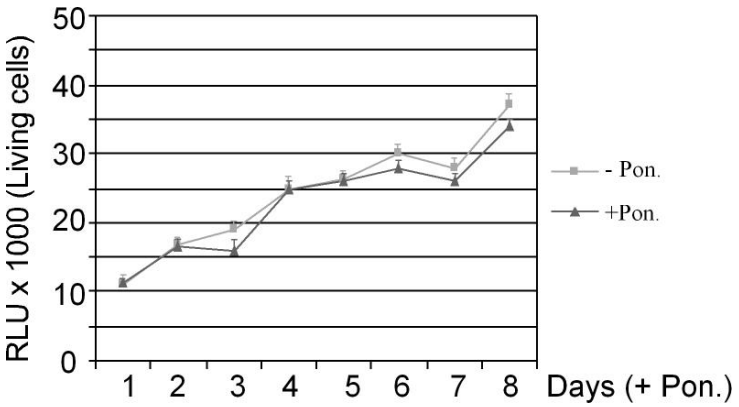


Figure 38: Growth properties of EcR293 cells.
The cells treated with (+) or without (-) ponasterone A (5 µM) throughout the time of the experiment. At the indicated time points, cells were measured with Cell-Titer Glo viability assay.

B. EcR293-NOS2 #33

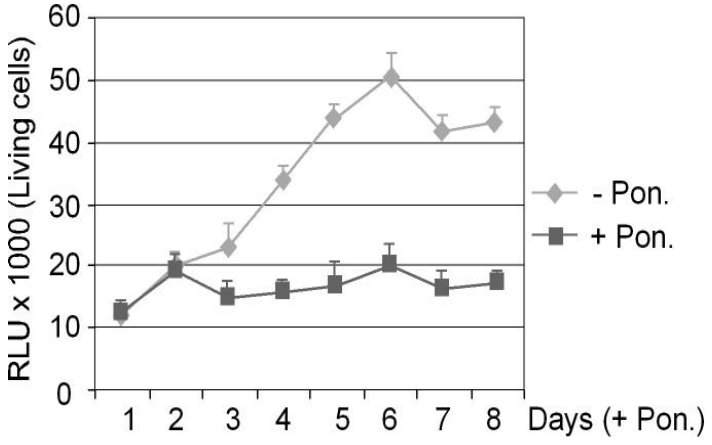


Figure 39: Growth properties of EcR293-NOS2 cells.
The cells were treated with or without ponasterone A (5 µM) throughout the time of the experiment and the cells were measured with luminescence viability assays at the indicated time points.

C. Growth properties of EcR293 and EcR293-NOS2 #33 cells

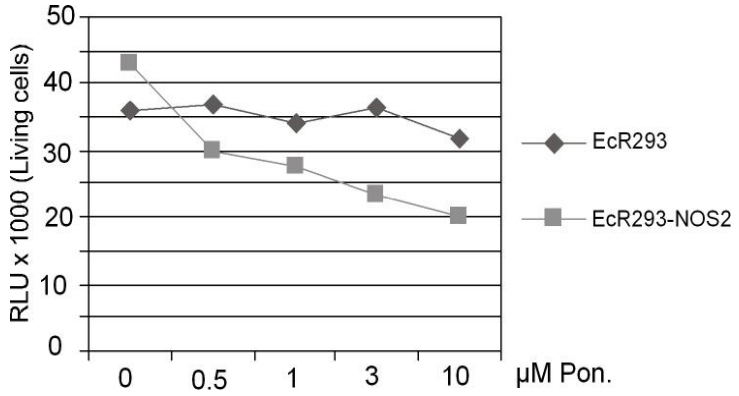


Figure 40: Growth properties of EcR293 and EcR293-NOS2 #33 cells treated with different concentrations of ponasterone A for 8 days and the viability measured. Experiments were performed in quadruplicates and mean values were calculated and plotted. Error bars indicate standard deviations.

D. Apoptosis in EcR293-NOS2 #33 after prolonged NOS2 induction.

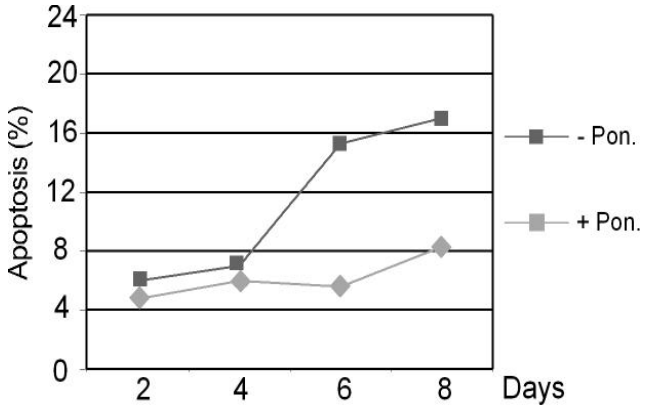


Figure 41: Apoptosis in EcR293-NOS2 #33 after prolonged NOS2 induction. Apoptotic sub-G_1 cells were quantified by FACS analysis.

Figure 38 showed the growth curves of EcR293 cells in the presence or absence of 5 µM ponasterone A. In this experiment there was no apparent differences in the cell numbers of the control EcR293 cells treated with (+) or without (-) ponasterone A for nearly 8 days culture. However, induction of the expression of NOS2 in EcR293-NOS2 cells led to drastic reduction in cell number compared to cultures left untreated

(Figure 39). To ensure that the growth retardation is dependent on the expression level of NOS2, EcR293 and EcR293-NOS2 were treated with various doses of ponasterone A for 8 days, and then measured the cell viability. Compared with untreated controls (- Pon.), the treated cells (+ Pon.) did not influence the viability of the EcR293 cells, whereas in EcR293-NOS2 cells a dose-dependent reduction of the cell viability reaching 50 % reduction with 10 μM ponasterone A was observed (Figure 40). From this experiment, it was demonstrated that the striking growth reduction was due to the effect of NOS2 expression in EcR293-NOS2 cells.

3.4.2 Endoplasmic reticulum stress and NO-induced apoptosis

3.4.2.1 Endoplasmic reticulum (ER) stress induced by NO

To explore the mechanisms underlying the induction of apoptosis in cells exposed longer periods to NO, endoplasmic reticulum stress and the influence on mitochondria membrane potential were investigated under the expression of NOS2 in EcR293-NOS2 cells.

3.4.2.2.1 ER stress from NO same as thapsigargin (an ER-stress stimuli)

Thapsigargin can induce ER stress through depletion of lumenal calcium stores in ER. For exploring the mechanisms underlying the induction of apoptosis in NOS2-expressing cells under long term exposure to NO, NOS2 expression was induced by ponasterone A at the concentration of 0, 1, 3, 10 mM and treated by thapsigargin (0, 0.5, 1, 2.5 μM). After treatment for 24 h with thapsigargin, Cell-Titer Glo viability assay was performed. The data were collected and analysed in Excel software.

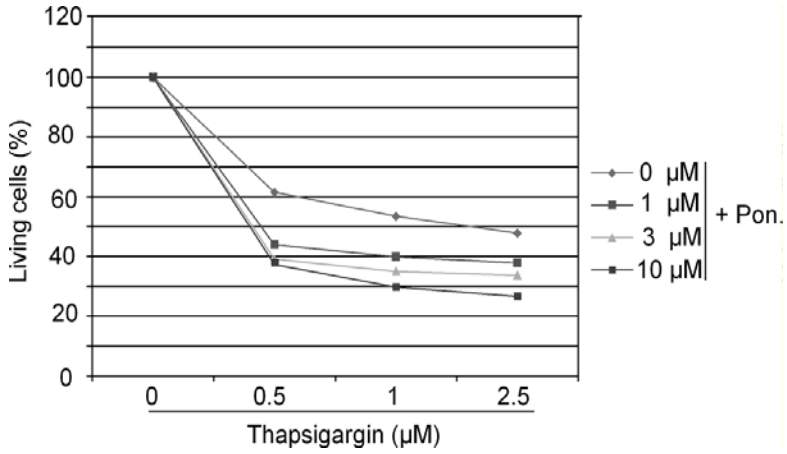


Figure 42: Influence of thapsingargin on the endoplasmic reticulum stress was enhanced by endogenous NO in EcR293-NOS2 cells. The cells were induced by 0, 1, 3, 10 µM ponasterone A for 24 hours and 0, 0.5, 1, 2. µM thapsigargin were used for this experiment.

Figure 42 shows that comparing the untreated cells (0 µM thapsigargin and 0 µM ponasterone); all samples treated by thapsigargin (from 0.5 µM to 2.5 µM) displaying reduced viability indicating ER stress in the cells. 2.5 µM thapsigargin lead to about 50 % of cell reduction under 0 µM ponasterone A induction. The cytotoxic effects of thapsigargin increased with higher levels of NOS2 suggesting that ER-stress was enhanced in the presence of NO.

3.4.2.2.2 BiP expression under ER stress

Accumulation of misfolded proteins or other ER stress related factors induces the expression of the ER chaperon BiP (GRP78/78 kD glucose regulated protein). For investigating the expression of BiP under ER stress from NO, EcR293-NOS2 cells were seeded in 6-well plates, induced by ponasterone A at the concentration of 0, 0.5, 1, 3 µM for 48 h. The cells were harvested, and 40 µg proteins were loaded on 8 % SDS-PAGE gel for Western blot analysis.

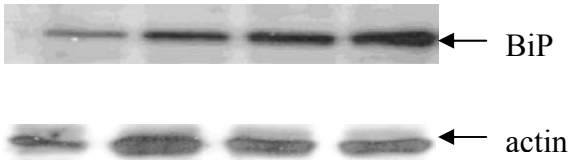


Figure 43: BiP expression was induced by NOS2 expression in EcR293-NOS2. Actin was used as control for the expression.

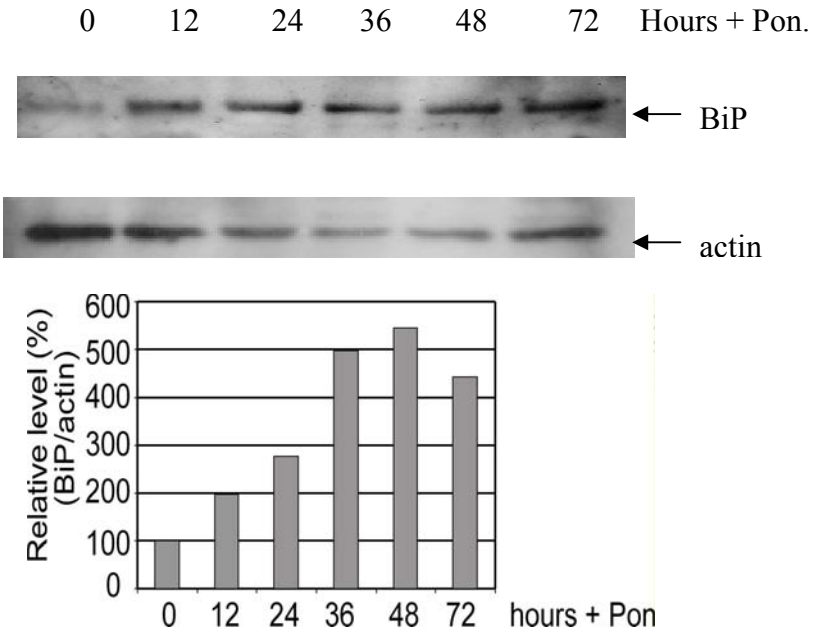


Figure 44: Time kinetic of BiP expression in EcR293-NOS2 #33 cells after NOS2 induction. Cells were induced with ponasterone A with 5µM ponasterone A for the designated times before Western blot analysis. Relative level of BiP/actin expression was analysed by Densitometer.

EcR293-NOS2 cells were treated with increasing concentrations of ponasterone A to induce NOS2-expression and then Western blot analysis was performed for the influence of the expression level of the ER chaperone BiP (Grp78). In untreated cells, low levels of BiP were detected. As the time of NO exposure increased, the amount of BiP increased (Figure 43 and 44) indicating NO-stress induced BiP-expression.

3.4.2.2.3 Immunostaining and Western blot analysis of the expression of CHOP/GADD153 and Western blot analysis of p53, ALG-2 and VCP expression

For exploring the expression of CHOP, a transcription factor induced by ER stress, under NO conditions, immunostaining and Western blots were performed. EcR293-NOS2 #33 cells were seeded in the 8-chamber slide, induced by 5 µM ponasterone A for 48 h, and then the cells were washed by PBS and treated with anti-CHOP antibody. Finally they were stained by Hoechst dye, and the samples were imaged by confocal laser scanning microscope.

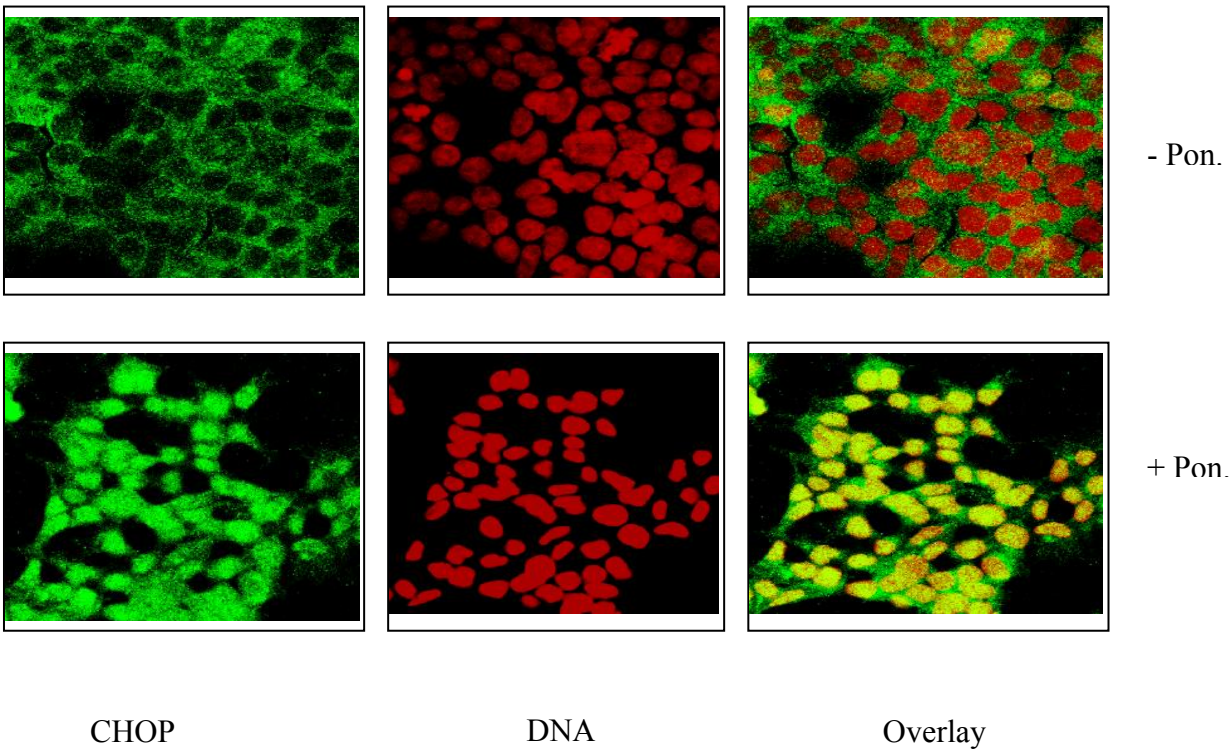


Figure 45: Confocal laser scanning image of EcR293-NOS2 #33 cells with NOS2 induction (+Pon) and without induction (-Pon) stained for CHOP (green). CHOP localizes primarily to cell nuclei (red) after induction. Overlay shows co-localization of CHOP and stained nuclei as yellow in lower right panel.

Images in Figure 45 show that the expression of transcript factor CHOP was induced by endogenous NO and indicated that ER-stress from NO induction induced CHOP expression in EcR293-NOS2 cells.

For Western blot analysis of p53 expression and the expression of ER stress-associated proteins or factors, such CHOP, ALG-2 and VCP in EcR293-NOS2 cells, the cells

were seeded in the 6-well plates and NOS2 expression was induced by the conditions showed in the Figure. Finally the cells were washed by PBS and harvested with 1 % NP-40 solution. 30 µg proteins were used and loaded on a 10 % SDS-PAGE gel to run.

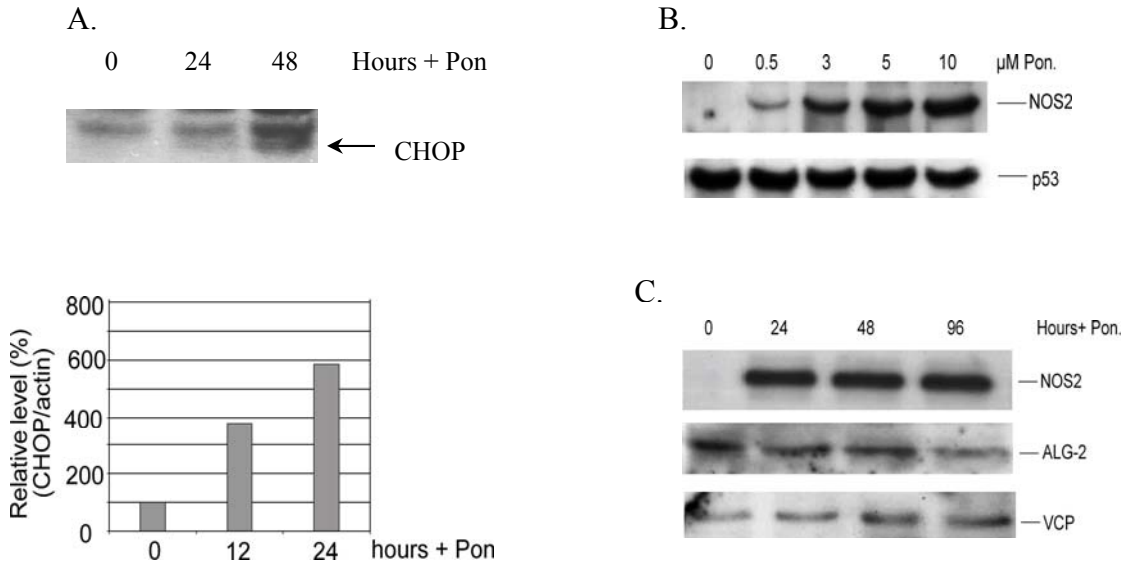


Figure 46: A. The level of CHOP expression in EcR293-NOS2 #33 cell was analysed by Western blot. The relative level of CHOP/actin was measured by Densitometer. B. The expression of p53 was not influenced by NOS2 expression. C. The expression of ALG-2 and VCP were not influenced by NOS2 expression in EcR293-NOS2 cells.

The result in Figure 46A showed an increased expression of CHOP after induction of NOS2 expression in EcR293-NOS2 cells. Comparison with uninduced cells (0 µM Pon.), the cells induced for the expression of NOS2, showed an increasing expression of CHOP. The expression of another 2 ER associated proteins, ALG-2 and VCP, were not influenced by NOS2 expression in Figure 46B and 46C. Compared to the control (0 µM Pon. or 0 hours + Pon.), the expression of ALG-2 and VCP were not changed after NO induction.

3.4.2.2.4 Caspase -4 activation in ER stress

Caspase-4 mainly is located in the ER and responsible for ER-stress induced apoptosis. It was tested that if caspase -4 was involved in NO induced ER stress in EcR293-NOS2 cells. The cells were seeded in the 6-well plates and induced by ponasterone A for 0, 24, 48, 72, 96, 120 h. The medium were removed and the cells were harvested. 30 µg

protein was used for caspase-4 activation assay, the samples were measured for proteolytic results.

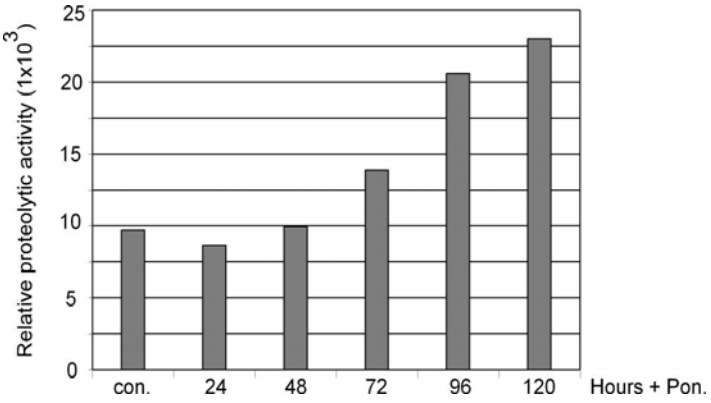


Figure 47: Caspase -4 activity was induced after NOS2 expression in EcR293-NOS2 cells
The cells were induced by 5 μM Ponasterone A for the designated times and Caspase-4 activity was measured with a fluorescent caspase substrate.

Figure 50 showed that caspase-4 activity was increased along with the increasing expression of NOS2 induced by ponasterone A in this time course. Together the expression of CHOP, BiP and caspase-4 activity, it indicated that ER stress was induced by the expression of NO in EcR293-NOS2 cells.

3.4.3 The influence of NO on mitochondria

3.4.3.1 JC-1 staining

For investigating the influence of NO on mitochondria, the change in mitochondrial membrane potential under long term exposure to NO was analysed by JC-1 staining. EcR293-NOS2 #33 cell were seeded in the 6-well plate and induced by ponasterone A at 10 μM for 3 days. Afterwards the cells were stained by JC-1 and measured by FACS.

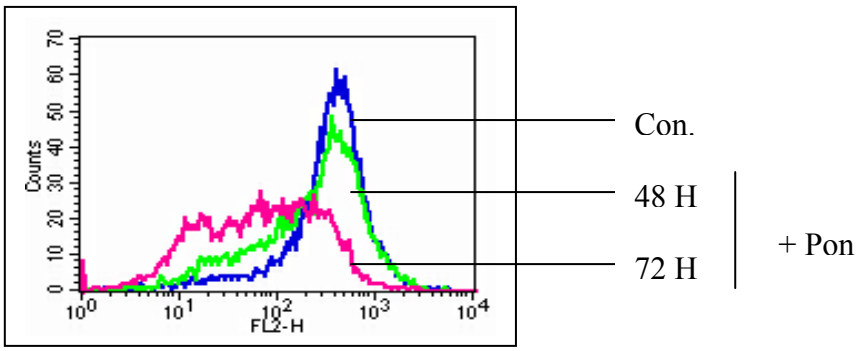


Figure 48: JC-1 staining shows a time dependent loss of the mitochondrial membrane potential in cells where NOS2 is induced by ponasterone A for 48 and 72 h

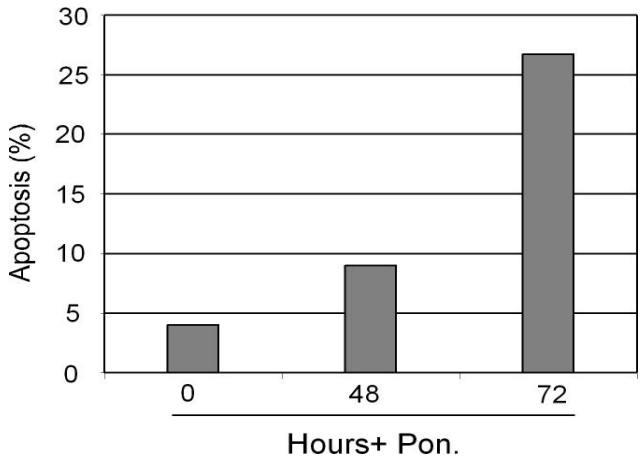


Figure 49: Loss of mitochondrial membrane potential during long term NO expression

Figure 48 and 49 show that loss of mitochondria membrane potential was observed after long time exposure to NO in EcR293-NOS2 cells. It indicated that long term exposure to NO resulted in extensive mitochondrial membrane potential loss without additional apoptosis stimulation.

3.4.3.2 Cleavage of caspase-9 under NO stress on mitochondria

Western blot analysis was performed for caspase -9 cleavage. EcR293-NOS2 #33 cell were seeded in the 6-well plates and induced them by ponasterone for 0, 12, 24, 36, 48

and 72 h. Then the cells were and 40 µg proteins were loaded for Western blot and anti-caspase -9 polyclonal antibody was used.

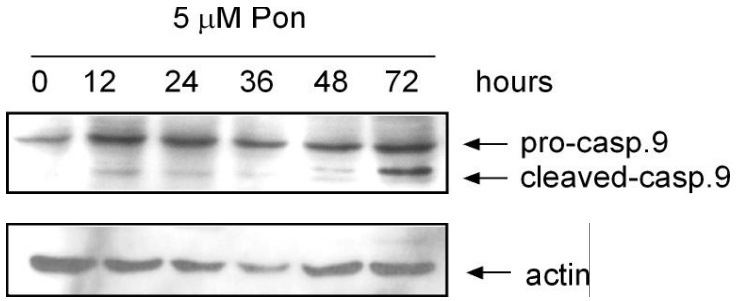


Figure 50: The cleavage of caspase-9 was analysed after the expression of NO induction without any stimulation.

The cleaved form of caspase -9 (Figure 50), which is associated with mitochondria-dependent apoptotic signalling pathway, was significantly observed in Western blot after NOS2 expression for 72 hours in EcR293-NOS2 cells.

Together these results of ER stress and cytotoxicity in mitochondria show that endogenously produced NO activates an ER- and mitochondrial-stress pathway which trigger apoptosis.

3.5 Selection of NO-resistant clones

If long time NO-exposure induces apoptosis, than all NOS2-expressing cells should be eliminated or counteract this death stimuli by acquiring apoptosis resistance. To test this hypothesis EcR293-NOS2 cells were cultured in the presence of 10 µM ponasterone A to continuously produce NO (medium containing G418 300 µg/ml , Zeocin 300 µg/ml). Significant cell death was observed in these cultures but cell colonies grew which were selected for further cultivation. After approximately three months of culturing in the presence of ponasterone A, the cell clones were analyzed for NO production by Griess assay.

3.5.1 Griess assay of NO production

For exploring NO production in surviving cell clones, different NO-resistant clones were seeded in the 6-well plates, and they were induced by ponasterone A at 5 μM for 48 h. Afterwards the medium was collected and the cells was harvest for Griess assay

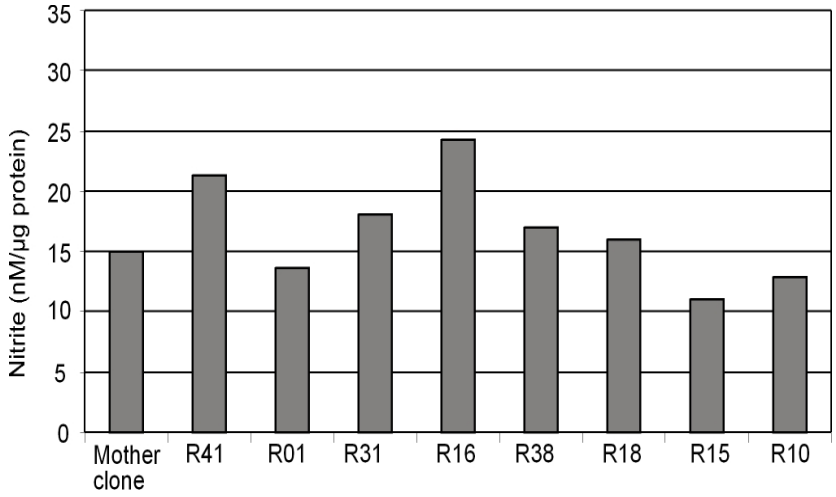


Figure 51: NO-resistant clones produce NO. The media from cells, resistant for continuous NO-stress were analysed for nitrite concentrations by griess assay.

Griess assay of NO production shows in figure 51 that all the cell clones which survived cultivation in the presence of 10 μM ponasterone A, also produced NO. These results demonstrate that the survival of the colonies was due to NO resistance and not merely to loss of ponasterone A inducible NO production.

3.5.2 Apoptosis sensitivity

To assess the effects of the NO-resistant clones on apoptosis, cells were seeded in the 12-well plates, they were induced by 5 μM ponasterone, after 24 h induction, the cells were treated by anti-APO-1. Apoptosis was analyzed by quantification of sub-G_1-cells on FACS.

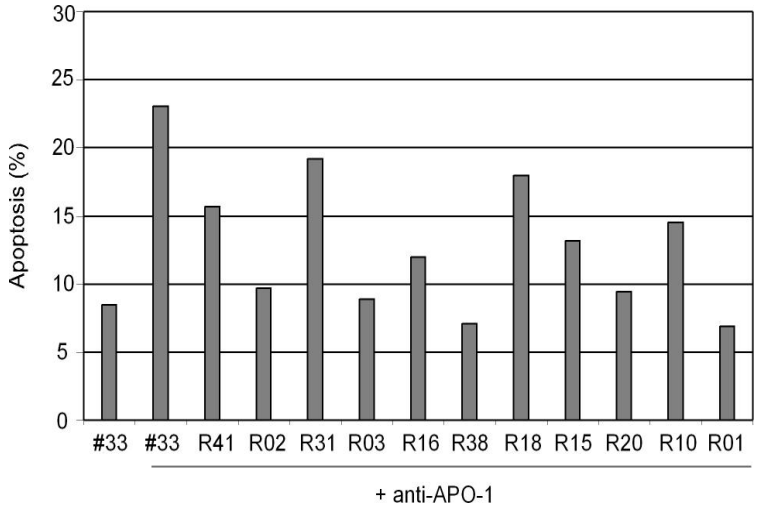


Figure 52: CD95-mediated apoptosis is attenuated in NO resistant EcR293-NOS2 cell clones. Induced cells were treated for 24 hours with 400 ng/ml anti-APO-1 and apoptotic sub G1-cells were quantified by FACS analysis.

The results in Figure 52 show that NO-resistant clones were cross resistant to CD95 mediated apoptosis induction even the expression of NOS2 was induced by ponasterone A.

3.5.3 Reduction of CTL killing

Based on the reduction of apoptosis sensitivity in NO-resistant cells, the influence of NO on the cytotoxicity of CTL was further investigated in these cells. The cells were seeded in the 6-well plates and induced them by ponasterone A according to different experiment conditions.

An allo-reactive A2.1-specific polyclonal CTL (CTL CD8 Allo-A2) was established as reported (Theobald, Biggs et al. 1995) for the Flu M1 58-66 peptide (CTL CD8x $A2K^b$ FluM1) has been described previously (Drexler, Antunes et al. 1999). CTLs were cocultured with ^{51}Cr labeled EcR293-NOS2 cells at the indicated effectors to target (E:T) ratios. ^{51}Cr release was measured after 4.5 h. The peptide specificity of the CD8 x $A2K^b$FluM1 CTLs was controlled by their failure to lyse target cells loaded with an irrelevant peptide.

A.CTL: CD8xA2k^b Flu

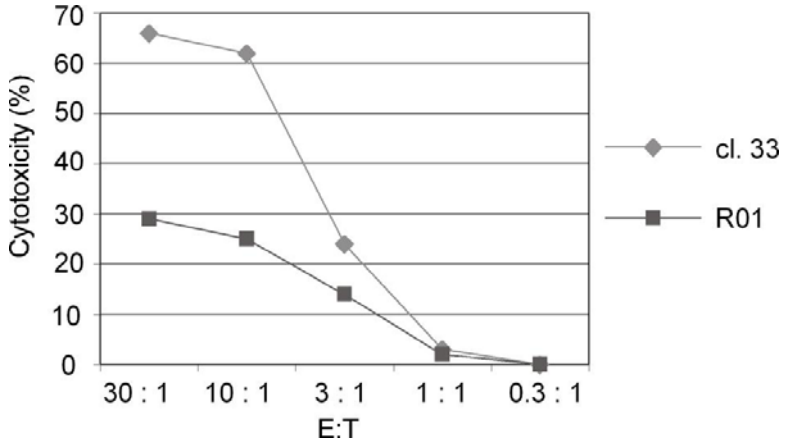


Figure 53: CTL-mediated apoptosis is decreased in NO-resistant clones R01. Coculture experiments were performed with allogenic or peptide specific T-cells CD8 allo A2 and EcR293-NOS2 #33 or NO-resistant clones R01

B.CTL: CD8xA2k^b Flu

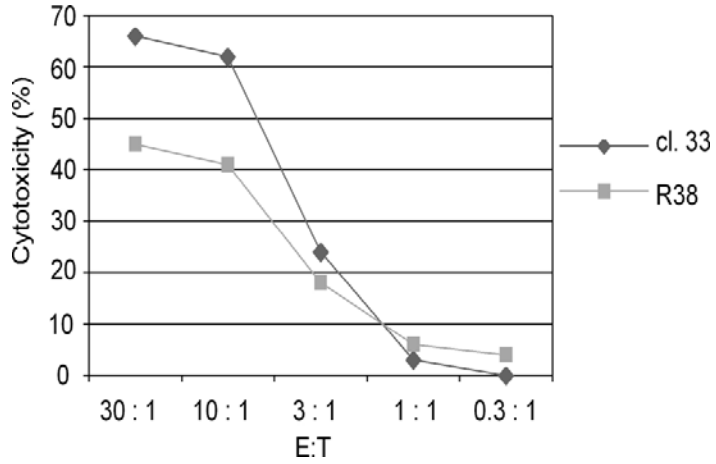


Figure 54: CTL-mediated apoptosis is decreased in NO-resistant clones R38. Coculture experiments were performed with allogenic or peptide specific T-cells CD8 allo A2 and EcR293-NOS2 #33 or NO-resistant clone R38

C. CTL: CD8 allo A2

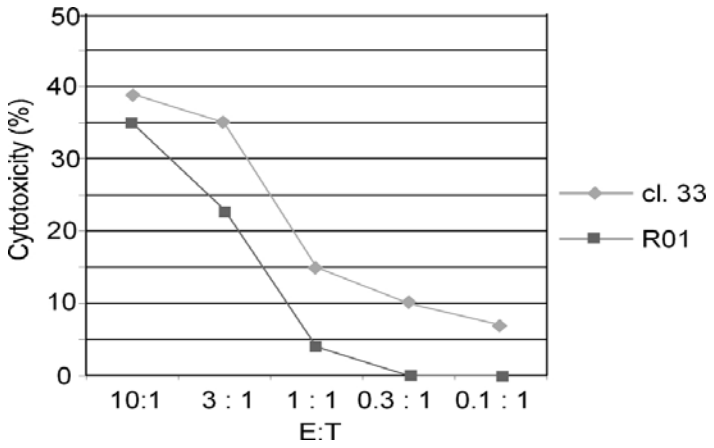


Figure 55: CTL-mediated apoptosis is decreased in NO-resistant clones R01 Coculture experiments were performed with allogenic or peptide specific T-cells CD8xA2k^b FluM1 and EcR293-NOS2 #33 or NO-resistant clone R01

D. CTL: CD8 allo A2

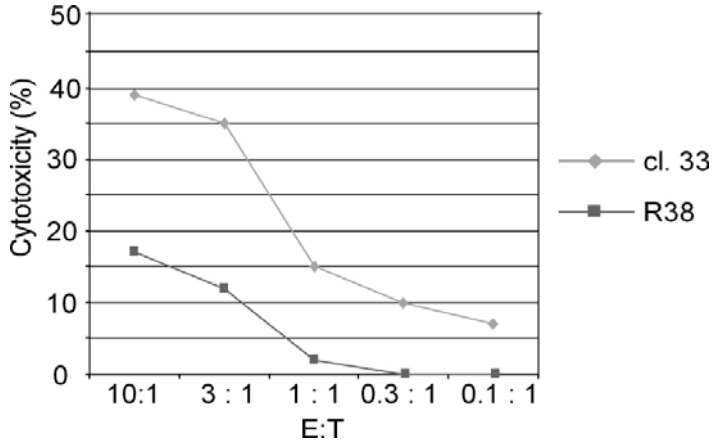


Figure 56: CTL-mediated apoptosis is decreased in NO-resistant clone R38 Coculture experiments were performed with allogenic or peptide specific T-cells CD8xA2k^b FluM1 and EcR293-NOS2 #33 or NO-resistant clone R38

The results in Figure 53, 54, 55 and 56 show apoptotic sensitivity mediated by different peptide specific CTLs were decreased in NO-resistant clone cells comparing with EcR293-NOS2 #33. Even when the expression of NOS2 was induced by ponasterone A, cocultured experiments with different peptide specific cytotoxic T cells demonstrate an acquired resistance to CTL-killing.

4. Discussion

As a free intracellular molecule, nitric oxide has various physiological and pathological functions in cells, such as antibacterial, defending pathogens and DNA damage. Lack of expression of nitric oxide synthases in knockout mice showed that this disruption in mice was not embryonic-lethal, but each (eNOS, NOS2, nNOS) showed selective pathological effects correlated with their biological functions of NO (Lala, 1998). For instance, eNOS knockout mice have hypertensive disorder (Huang, 1995), because NO produced by eNOS from endothelial cells functions as a vasorelaxant role in the vessels. The NOS2 knockout mice ($NOS2^{-/-}$) was easily infected and showed inability of macrophage killing functions against parasites and tumor cells (MacMicking, 1995), while NO produced from macrophage play an important role in anti-pathogens, bacterial or anti-tumor cells. The nNOS knockout mice ($nNOS^{-/-}$) reflected a hypertrophic pyloric stenosis consistent with the disrupting neurotransmitter role of NO in relaxation of pyloric sphincter muscles (Huang, 1993). Of those three NO synthesis enzymes, the inducible NOS (NOS2) isoform has crucial role in immune defense in the body, because of the large quantity of NO was produced by the macrophage NOS2 (Nussler, 1993).

However the effects of nitric oxide (NO) in the cells are controversial. On one hand studies indicate that nitric oxide (NO) produced from NO donors like S-Nitroso-N-acetyl-D, L-penicillamine (SNAP) in vitro or in vivo from NO synthesis genes may promote cell death (Burney, 1999; Burney, 1997; Huerta-Yepez, 2004; Li, 2004; Rodriguez-Lopez, 1999). On the other hand, other researche has suggested that NO may have an anti-apoptotic role (Kim, 1997; Siegert, 2002; Wenzel, 2003), because many tumors, such hepatocellular carcinoma and colon cancers express high levels of NOS2 (Cianchi, 2003; Daghigh, 2002; Vakkala, 2000; Wolf, 2000). While chronic inflammation is related with the induction of NOS and the NOS2 enzyme can efficiently produce NO (Tozer, 1997); it was suggested that NO can trigger tumor development by functioning as endogenous mutagen, an angiogenesis factor, a mitogen, an enhancer of proto-oncogene expression (Suschek, 1999) and one factor of anti-apoptosis (Ohshima, 1994), this display an apoptosis resistant phenomenon and tumor cells may increase the possibility of escaping immune surveillance.

Short term NO effects

4.1 Endogenous produced NO enhances CD95- or TRAIL-mediated apoptosis sensitivity

Through FACS analysis, caspase activity assay and JC-1 staining of mitochondria membrane potential change, the present study demonstrate that endogenously produced NO from NOS2 expression play a pro-apoptotic role in both CD95- and TRAIL-induced signalling pathway in EcR293 cells.

Recently it was reported that NO-mediated apoptosis sensitivity was involved with tumor suppressor gene p53 and mitochondria in both CD95- and TRAIL-induced signalling pathway, the accumulation of p53 gene was detected and the depolarization of mitochondria was observed in the cells (Green, 1998; Duchen, 2000; Forrester, 1996; Calmels, 1997; Hofseth, 2003). Mitochondria play a crucial role in apoptotic effects through release the proapoptotic factor, cytochrome c, Smac, apoptosis-inducing factor (AIF) (Joza, 2002). The proteins from Bcl-2 family including both anti-apoptosis members, such as Bcl-2 and Bcl-xL, and proapoptotic members like Bax, Bak, Bid were also involved in cell death signalling pathway and regulates the intergrity of mitochondrial membrane (Joza, 2002; Scorrano, 2003). During the apoptosis process, changing of p53 gene may regulate the expression of many downstream genes that mediates DNA repair, cell cycle or mitochondrial membrane permeability, therefore the release of proteins from mitochondria is also related with the accumulation of p53 gene (Moll, 2001).

It is reported that NO promotes apoptosis and inhibits growth of cancer through activating tumor suppressor gene p53 (Hofseth, 2003; Messmer, 1996; Forrest, 1996). However in present study, the p53 gene accumulation was not found after the expression of NOS2 in EcR293 cells (Figure 46), even the loss of mitochondrial membrane potential ($\Delta\Psi_m$) was observed by JC-1 staining in both CD95- and TRAIL-treated cells. Maybe in EcR293 cells, the augmentation of apoptosis sensitivity from NO was independent of p53 regulation. In both CD95- and TRAIL-induced cells, the caspase-8, -9 and -3 activations were observed. And under the induction of NOS2, the activation of those caspases were much high than in NOS2 unexpressed cells. Some studies found that the apoptosis sensitivity enhanced by NO was related with an

increasing expression of the CD95 receptor on the cell membrane (Garban, 1999; Fukuo, 1996). Based on these finding, it was investigated whether the expression of CD95 or TRAIL receptor was increased by endogenously produced NO. However the expression of CD95 and TRAIL receptors on the cell membrane were not influenced by the expression of NOS2 in present study through Western blot and FACS analysis. This indicated that other factors may be influenced by NO and then enhanced the apoptosis sensitivity in both CD95- and TRAIL-mediated apoptosis signal.

4.2 Endoplasmic reticulum and mitochondria were involved in the NO-mediated apoptosis signal

High levels of sustained ER stress may result into programmed cell death or apoptosis, and some proteins or factors involved have been identified. Caspase -12, an ER-associated cell death effecter, is specifically activated by proteolytic processing during ER stress in the mouse. ER stress can induce cell death through caspase-12 activation pathway. Another effecter is CHOP/GADD153, one ER-related proapoptotic transcription factor, NO-induced apoptosis in mice occurs through the ER stress pathway involving CHOP (Kawahara, 2001). CHOP is significantly up-regulated during ER stress process. Some studies demonstrated that the CHOP gene is induced by ER stress through a signalling events that involved with the pancreas ER kinase(PERK) activation and phosphorylation of the translation initiation factor eIF2α. The CHOP gene encodes a transcription factor that promotes programmed cell death (Zinszner, 2000, McCullough, 2001).

But human caspase -12 protein is dysfunctional even though it is transcribed and expressed, because of a frame shift creating a stop codon which leads to a inactive protein. It was reported that human caspase-4 is a homologous protein of the rodent's caspase -12. Using different analysis method they showed caspase -4 was mainly localized to endoplasmic reticulum membrane, and possibly to the mitochondria membrane. They verified ER-stress induced apoptosis involves caspase -4 (Hitomi, 2004).

In the present study, using immunofluorescent staining and Western blot, it was found that the expression of GADD153/CHOP gene is induced under conditions where NOS2 was expressed in EcR293 cells, but the control cells or NOS2 uninduced cells showed much lower or undetectable GADD153/CHOP suggesting ER stress was involved. The human caspase -4 activation is also observed under the expression of NOS2 after a few days induction by ponaterone A in the cells and the increased expression of ER stress chaperone BiP is also detected by Western blot. Those factors are closely related with ER stress-associated apoptosis or cytotoxicity in cells. From these experiments, it is significantly different in the expression or induction between the NOS2 expressing cells or non-expressing cells. It suggests that the cytotoxicity of NO are involved with factors or proteins associated with ER-stress in cells. The expression of NOS2 may induce the signalling pathway related with ER stress-associated factors or proteins, and it may not only up-regulate GADD153/CHOP, but also influence the proteolytic signalling way through caspase -4 or BiP protein.

Valosin-containing protein (VCP) and apoptosis-linked gene-2 (ALG-2) were reported association with ER stress in the cells (Ye, 2001; Rao, 2004); VCP from ER stress induction may induce the cell into apoptosis. However the difference of VCG protein or ALG-2 in EcR293-NOS2 cell clones was not detected after the induction of NOS2 by ponasterone A. Perhaps the ER additional tress signal pathways are involved.

Mitochondria play a center role in the cytotoxicity in cells. As the energy resource for the cell, any change of mitochondria in the respiration chain, ion channel or membrane potential will be harmful for the cells and may induce apoptosis.

In the present experiments, it was found that long term exposure to NO induces the loss of mitochondrial membrane potential ($\Delta\Psi_m$). And FACS analysis showed that there was a clearly apoptosis in the cells under long term NO stress compared with the control cells. Caspase-9 emerges as the main caspase related with mitochondria pathway in the apoptosis signal. The cleavage of caspase-9 was found in Western blot under long term NO stress in these experiments. The activation of caspase-9 may trigger other downstream signalling substrates and activate the important apoptotic effectors caspase-3 and finally induced cell death.

In present investigation, it was found that the endogenously produced NO from the expression of NOS2 led to striking cell growth reduction in EcR293-NOS2 after induction by ponasterone A, but no significant alternation in the cell cycle was observed in EcR293 cells under ponasterone A treatment.

Collectively the results of long term NO stress strongly suggested that the cytotoxicity of endogenously produced NO may depend on mitochondria and endoplasmic reticulum signalling pathway. And ER stress associated proteins, CHOP/GADD153, BiP and caspase-4 and the loss of $\Delta\Psi_m$ in mitochondria under NO stress, cleavage of caspase-9 were tightly involved in NO cytotoxicity in EcR293-NOS2 cell lines.

4.3 NO augments CTL-mediated apoptosis

Cytotoxic T lymphocytes (CTLs) are derived from immune activation of T cytotoxic (Tc) cells. These effector cells have lytic capability and are important in the recognition and removal of noself-cells (such as tumor cells and virus-infected cells). Normally CTLs are $CD8^+$ cells, and these $CD8^+$ CTLs have been described to play a crucial role in host defense against malignancies in both mouse and human. In CTL - mediated cytotoxicity process, two major pathways are engaged following TCR recognition of Ag/MHC complexes expressed on target cells (Thiery, 2005*).* The first phase is a secretory pathway involving receptor-triggered exocytosis to activate and differentiate naive Tc cells into functional effector CTLs. The second is based on receptor-induced surface expression of death receptor ligands on effector cells, which cross-links the corresponding receptors such as CD95 on target cells and induced them to destroy the target cells. This death induction is dependent on the death receptor and ligand system. Another way for initiating CTL-mediated apoptosis and independent of the death receptor are the cytotoxic proteins such as perforin and granzymes which are released from CTLs and enter target cells to induce apoptosis.

The effect of NO on the CTL mediated apoptosis is still not fully understood. Evidence has begun to accumulate that macrophage-derived NO can also inhibit lymphocyte responses in vivo. NO could inhibit immune responses stands in contrast to the role of NO as an effector destroying of bacteria and tumor cells (Hibbs, 1987). Nevertheless other studies indicated NO could enhance T cell killing. In present experiment, co-

culture CTLs and target NOS2-expressing cell, endogenous NO enhanced the CTL-mediated killing. The apoptosis sensitivity induced by both CTL CD8xA2kb Flu and CD8 allo A2 was significantly increased by nitric oxide in EcR293-NOS2 cells. Dose-dependent induction of NOS2 in EcR293-NOS2 #33 cells correlates with apoptosis induced by cytotoxic T cells (CD8 allo A2) at E:T ratio 3:1.

Based on these results it could conclude that endogenous NO enhances the apoptosis sensitivity in CD95- or TRAIL-mediated pathway and increases the CTL-induced cell death. This mechanism is important for the immune surveillance.

Long term NO effects

4.4 NO-resistant clones shows also resistant to CD95- or CTL-induced apoptosis

Short term cytotoxic effects of NO have been documented; the possible targets being the ER and mitochondria. The present study demonstrates that one of the short term effects of endogenous NO production is to enhance sensitivity for the CD95-mediated apoptosis pathway. Prolonged exposure to NO induced ER- and mitochondrial stress causes activation of caspase-4 and -9 leading to apoptosis. Furthermore, cytokines, secreted by lymphokine-activated killer cells can induce endogeneous NO-synthesis and apoptosis in colon cancer cells. This non-contact-dependent cell toxicity mechanism supports a role for NO in T cell mediated tumor defense.

These results highlight a paradoxical role of NO during tumorigenesis. On one hand, NO induces apoptosis and sensitizes cells to apoptosis mediated by CD95 or TRAIL, and on the other hand, NO promotes survival of tumor cells. Based on these results, It was hypothesized that that the apoptosis-enhancing effect of NO initially reduces tumor formation but finally results in the elimination of cells with a fully functional apoptotic response and the retention of a subpopulation of cells with an aberrant or attenuated response to death-inducing signals. Chronic exposure to NO thus facilitates the clonal evolution of a population of cells that can circumvent normal death inducing signals, including those derived from CTLs during immune surveillance. One of the hallmarks of cancer cells is apoptosis resistance. Selection for apoptosis resistant tumor cells may be the product of repeated genomic and cellular insults due to continuous NO exposure. Analysis of the cell clones obtained during these

experiments may reveal the molecular mechanisms involved in developing NO and apoptosis resistance in tumor cells.

4.5 The indication for tumorigenesis

Neoplastic cells can escape or resist the immune response at multiple levels. Accumulating evidence suggests that in vivo tumor cell growth is not only influenced by CTL tumor cell recognition, but also by the susceptibility of the tumor cells to host-mediated immune response.

In the experiments with long term NO exposure, it was found that long term exposure to endogenously produced nitric oxide (NO) could result the cells to growth arrest or significantly reduction of cells growth, and long term exposure to NO could also lead to the cells undergoing apoptosis, which mainly involved mitochondria and endoplasmic reticulum. The process included the loss of mitochondria membrane potential and induction of several ER stress-associated factor in endoplasmic reticulum, such GADD135/CHOP, BiP and ER specific caspase-4 in human. Furthermore the continuous exposure to endogenously produced NO selectively led to cells resistance to NO toxicity. This phenotype of cells also demonstrated a cross resistant to CD95-induced apoptosis and also showed resistant to CTLs-killing (here CD8xA2kb Flu and CD8 allo A2 CTLs were used).

It was reported also that NO can protect the astrocytes from apoptosis through switching on glycolysis to provide energy for the cells (Angeles, 2004), this response was not available in neurons. The production of NO by the induction of NOS2 was common in many tumors. Human tumors such as melanoma, bladder cancer, breast cancer, hepatocellular carcinoma and colorectal cancer expressed high levels of NOS2. NO from the expression of NOS2 in tumors can provide an advantage aspect to protect tumor cells from death (Kim, 1997). In immunological concepts tumors were developed only if the tumor cells are able to escape from the immune surveillance. CD95 receptor/ligand system plays crucial role in the immune surveillance by eliminating tumor cells through CD95-mediated apoptosis. Lack of the expression of CD95 receptor on the cells surface or blockage of downstream signal pathway may lead to tumor cells resistant to CD95-mediated apoptosis. Human tumors such as renal

cell carcinomas, gastric carcinomas or pancreatic cancer escape from immune surveillance through the lack of CD95 receptor expression on the cell surface.

Many studies have been reported that NO play an important role in tumor progression in animal models (Kennovin, 1994; Orucevic, 1996; Edwards, 1996; Dong, 1994). Inhibition of NOS expression reduced NO production and tumor growth and NOS2 expression in human colonic adenocarcinoma cell line was associated with increased tumor growth (Jenkins, 1995). Furthermore, tumor derived NO was shown to promote tumor cell invasiveness and a suppression of host anti-tumor defense (Lala, 1998).

In present experiment model, the expression of CD95 receptor was not influenced by the induction of NOS2 in the EcR293-NOS2 cells in either short term or long term exposure to endogenously produced NO. However, once the cells are resistant to NO under long term exposure to NO, these resistant clones also showed cross resistance to CD95- or CTLs-induced cell death. These results suggest a possible mechanism how the tumor cells escape from immune surveillance through the induction of the expression of NOS2, especially the immune surveillance and how NO may contribute to tumor progression. Namely tumor cells, expressing NO are selected for resistance to NO toxicity and further achieve resistance to CD95- or CTLs-induced apoptosis.

5. Summary

NO is associated with tumor development and progression. The cellular mechanisms by which endogenous NO exposure in tumor cells can modulate apoptosis sensitivity was investigated, giving particular focus on apoptosis mediated by the CD95 system. For this purpose, a NOS2 expression vector driven by an ecdysone-inducible promoter was constructed; the vector was then introduced into EcR293 cells. Ecdysone dependent NOS2 expression and NO production were detectable in a number of cell clones. Using this cell culture model, short and long term effects of endogenous NO exposure on CD95-mediated apoptosis was tested. The results demonstrate a dramatic enhancement of CD95- or TRAIL-mediated apoptosis during acute episodes, with increased caspase activity and mitochondrial membrane depolarization. Furthermore, in co-culture experiments the cytolytic action of T cells against NO-producing tumor cells is enhanced. Continuous long term exposure to endogenous NO initially reduced cell numbers and induced apoptosis. However, cell clones grew out that were resistant to both NO and apoptosis after chronic NOS2 expression.

Diseases associated with chronic inflammation predispose individuals to cancer. NO has attracted considerable attention as a possible link between inflammation and tumorigenesis for several reasons. First, due to its free radical nature NO is capable of reacting at different points in the cell by modifying proteins and DNA. Second, NO is produced during inflammation by induction of NOS2. Third, NOS2 expression is observed in many tumors and is continued to be expressed in advanced tumors suggesting that it has a role for tumor development (Thomsen L Br J Cancer, 72 1598-1610, 1995). Moreover, there is extensive experimental evidence to support a role for NO in accelerating tumor development but the cellular and molecular mechanisms by which NO promotes tumor formation and growth are not fully understood. Furthermore, the accumulation of DNA lesions during NO stress may be in part attributed to the inhibition of DNA repair enzymes by NO. (GORES) Thus, continuous genomic insults due to NO production may have negative effects on genome stability promoting tumor progression.

Short-term cytotoxic effects of NO have been clearly documented; the possible targets being the ER and mitochondria. The present study demonstrates that one of the short term effects of endogenous NO production is enhanced CD95- or TRAIL- signalling leading to apoptosis. Prolonged exposure to NO induced ER- and mitochondria-stress causing activation of caspase -4 and -9 leading to apoptosis. This study highlights a paradoxical role of NO during tumorigenesis. On one hand, NO induces apoptosis and sensitizes cells to apoptosis mediated by CD95, and on the other hand, NO promotes survival of tumor cells. On the basis of the results presented here, it was proposed that the apoptosis-enhancing effect of NO results in the elimination of cells with a fully functional apoptotic response and the retention of a subpopulation of cells with an aberrant or attenuated response to death inducing signals. Chronic exposure to NO thus facilitates the clonal evolution of a population of cells that can circumvent normal death inducing signals, including those derived from CTLs during immune surveillance. One of the hallmarks of cancer cells is apoptosis resistance. It was hypothesized that selection for apoptosis resistant tumor cells may be the product of repeated genomic and cellular insults due to continuous NO exposure. Analysis of the cell clones obtained during these experiments will further characterize the molecular mechanisms involved in developing NO and apoptosis resistance in tumor cells.

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7. Abbreviation:

µl	Microliter
AA/BA	Acrylamid/Bisacrylamid
APS	Ammonium persulfate
ATP	Adenosintriphosphate
NO	Nitric oxide
bidest	Bidestilled
BSA	Bovine Serum Albumin
Ca	Calcium
$CaCl_2$	Calciumchlorid
CIP	calf intestinal phosphatase
CLSM	confocal laser scan microscope
DNA	Desoxyribonucleinacid
DTT	Dithiotreitol
dTTP	desoxytyrosintriphosphate
EDTA	Ethylendiamin-N,N,N´,N´-tetraacetic acid
EGTA	Ethylenglycobis(2-amino-ethylether)-N,N,N´,N´-tetraacetic acid
FACS	fluorescence-activated-cell-sorter
GDP	Guanosindiphosphat
GFP	green fluorescence protein
GTPase	Guanosintriphosphatase
h	Hour
HCl	Hydrochlorid
HLA	human leukocyte antigen
IgG	Immunglobulin G

kD	kilo-Dalton
kV	kilo-Volt
LB	Luria-Bertani medium
Luc	Luciferase
mA	Milli-Ampere
MCS	multiple cloning site
MgCl	Magnesiumchlorid
min	Minute
ml	Milliliter
$MnCl_2$	Manganchlorid
mRNA	Messenger ribonucleinacid
NaCl	Natriumchlorid
NaOH	Natriumhydroxid
PAGE	Poly-Acrylamid-Gelelectrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pKS	Bluescript-Vector KS
rpm	rounds per minute
RT	Room temperature
SDS	Sodium Dodecyl Sulfate
TEMED	N,N,N´,N´-Tetramethylendiamin
Tris	Tris-(hydroxmethyl)-aminomethan
µg	Microgram
UV	Ultraviolett
v/v	volume/volume (Vol.%)
NOS2 (iNOS)	Inducible nitric oxide synthesase

TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
CD95L	CD95 ligand
Pon.	Ponasterone A