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The regulation of manganese superoxide dismutase and inducible nitric oxide synthase by nuclear factor-Kappa B and activator protein 1 in rat aortic endothelial cells

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The Regulation of Manganese Superoxide Dismutase and Inducible Nitric Oxide Synthase by Nuclear Factor-Kappa B and Activator Protein 1 in Rat Aortic Endothelial Cells

DISSERTATION

Submitted to the Graduate College

of

Marshall University

In Partial Fulfillment of the Requirements for

The Degree of Doctor of Philosophy

By

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Huntington,

West Virginia

2001

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To the memory of my grandmother Jessie Sypolt

and grandfather Herbert L Cobbs Sr.

Table of Contents

List of Tables and Figures

Abstract

Inducible nitric oxide synthase (iNOS) and manganese superoxide dismutase (MnSOD) are two enzymes that influence reactive oxygen species within the cell. The promoter regions for the genes encoding these enzymes have two specific transcription factor elements in common, activator protein 1 (AP-1) and nuclear factor kappa b (NF-KB). This study was initiated to determine if either NF-KB or AP-1 regulates the transcription of both genes in endothelial cells. To test whether NF-KB or AP-1 binding sites in the MnSOD and iNOS promoter region were important for transcriptional regulation, we performed induction analysis using various deletion constructs of MnSOD and iNOS luciferase reporter plasmids expressed in rat aortic endothelial cells (SVAREC and PRAEC). We found that the basal and inducible regulation of MnSOD are dependent upon the NF-KB binding site. Deletion of this ten base pair sequence produced a 90% decrease in basal expression and a 99% decrease in expression of lipopolysaccharide (LPS) treated cells, also studies with the inhibitors parthenolide and A-Fos, supported the finding that NF-KB is critical for MnSOD transcription. The AP-1 site is not necessary for basal transcriptional regulation of MnSOD but does seem to be necessary for induction of MnSOD, since deletion of the AP-1 binding site reduces reporter expression of LPS treated cells down to basal levels. Taken together, these results together suggest that MnSOD expression is dependent upon NF-KB and AP-1 for induction of transcription, while only NF-KB is necessary for basal levels of MnSOD transcription. Serial deletions and inhibitor studies of iNOS suggested that the NF-KB site between

–680 and –266 is important for iNOS transcription. All of this data suggests that MnSOD and iNOS are reliant upon the transcription factor NF-KB for induction of transcription.

1.0 Introduction

1.1 Nitric Oxide

The human body has a complex circulatory system to supply its organs with nutrients and oxygen. This system is comprised of the heart, blood, and the vasculature, which includes the arteries and veins. The blood carries oxygen to the organs through the vasculature by a force created by the heart. This force, known as blood pressure, produces a pressure in the arteries necessary to force the blood through the arteries and veins. The blood pressure averages 100mm Hg in the arteries and approaches zero in the veins. There are times when the body needs to regulate this pressure to increase or decrease blood flow to specific organs. If metabolic activity increases, the organs need more oxygen, metabolites are released, and blood volume increases to meet the higher oxygen demand. In order to fulfill this requirement, the arteries and veins can dilate or constrict, in processes called vasodilation and vasoconstriction, respectively. Vasodilation occurs when smooth muscle relaxation increases vasculature diameter. This process increases blood volume but decreases blood flow and blood pressure. Vasoconstriction occurs when the vasculature decreases in diameter to decrease blood volume and increase blood flow and blood pressure (Guyton, 1991). Humoral factors secreted by the body regulate vasoconstriction and vasodilation. Factors that stimulate vasoconstriction include

norepinephrine, epinephrine, angiotensin II, and vasopressin. In addition, there are many humoral factors that stimulate vasodilation, including bradykinin, histamine, certain prostaglandins (Guyton, 1991), and acetylcholine (ACh) (Moncada et al., 1991a).

Furchgott and Zawadzski (1980) demonstrated that ACh-induced vascular relaxation was dependent on an intact vascular endothelium. If the endothelium was removed, ACh treatment produced no vascular relaxation. The factor which mediates ACh-induced relaxation is known as endothelium derived relaxing factor (EDRF). Endothelium dependent relaxation occurs in response to several stimuli, such as ACh, adenine nucleotides, thrombin, substance P, the calcium ionophore A23187, and bradykinin (Moncada et al., 1991b).

The humoral nature of EDRF was demonstrated by using donor and detector bioassays. One system consisted of two rabbit aortic strips in which the donor aorta retained an intact endothelium and the detector strip had the endothelium removed. The EDRF donor aortic strip was placed next to the detector strip, intimal surface to intimal surface (Furchgott, 1984). ACh stimulation of the donor strip caused the relaxation of the detector tissue. In a second bioassay, an intact rabbit aorta, the donor, was perfused with ACh. The effluent was collected and superfused on to an endothelium denuded rabbit vascular ring (the detector). ACh stimulation of the donor tissue produced an effluent that could cause relaxation of the detector, providing additional evidence that EDRF is a humoral factor (Cocks et al., 1985). A second line of evidence for the humoral status of EDRF was based on vascular endothelial cells grown on microcarrier beads and perfused with ACh. The effluent was collected by column chromatography and used to superfuse

either canine coronary artery rings or denuded rabbit aortic strips. This effluent caused denuded aortic strips and canine coronary artery rings to relax (Cocks et al., 1985; Gryglewski et al., 1986a). Through experiments using different lengths of tubing to deliver the effluent from a donor tissue to the denuded aorta at varying time points, it was established that EDRF had a half-life of only a few seconds in physiological salt solutions (Griffith et al., 1984; Cocks et al., 1985).

The donor and detector systems suggested that EDRF was a factor produced by the endothelium and secreted into the blood. Studies in which the donor and detector were separated, allowed chemical manipulation of the generation, action, and stability of EDRF (Cocks et al., 1985; Rubanyi et al., 1986; Gryglewski et al., 1986a). It was found that superoxide anions (O_2^-) contribute to the instability of EDRF because administration of superoxide dismutase (SOD) into the donor detector system prolonged the effects of EDRF (Gryglewski et al., 1986b) and because compounds that generate O_2^- block relaxation by EDRF (Rubanyi and Vanhoutte, 1986). These findings suggested that O_2^- reacts with EDRF and prevents its biological action.

What is EDRF and how is it produced by the endothelium? Initially, EDRF was thought to be a product of the arachidonic acid lipoxygenase pathway (Singer and Peach, 1983; Fostermann and Neufang, 1984), or the cytochrome P-450 enzyme system (Pinto et al., 1986; Macdonald et al., 1986). Later, Furchgott (1988) and Ignarro et al. (1988) suggested that EDRF might be nitric oxide (NO) or a closely related species. The first evidence was the chemical detection of NO release from vascular endothelial cells. NO reacts with ozone to form a chemiluminescent product, which was used to quantitate the

amount of NO produced by the endothelial cells (Downes et al., 1976). This led to the discovery that bradykinin stimulation of endothelial cells induces EDRF release and NO production (Palmer et al., 1987), which were considered two separate events at this time.

A detailed comparison of NO and EDRF showed that the two compounds produced exactly the same effect on vascular strips (Palmer et al., 1987; Hutchinson et al., 1987) and platelets (Radomski et al., 1987b,c). EDRF and NO maintain relaxation of vascular strips, which decline at the same rate (Palmer et al., 1987). Both compounds also inhibit platelet aggregation (Radomski et al., 1987b) and platelet adhesion (Radomski et al.,1987c,d), and induce platelet disaggregation (Radomski et al., 1987b). The decay rates for both compounds were similar under in vitro conditions. The action of EDRF and NO on vascular strips and platelets is potentiated by SOD and cytochrome c and is inhibited by O_2^- generated by the redox compounds pyrogallol, dithiothreitol, and hydroquinone. Also, redox compound inhibition of EDRF and NO relaxation of the vasculature can be attenuated by SOD (Griffith et al., 1984; Palmer et al., 1987; Hutchinson et al., 1987; Radomski et al., 1987a). The sum of all this evidence supports the hypothesis that EDRF is NO.

1.1a Biosynthesis of Nitric Oxide

Although the identity of NO and its production by endothelial cells had been established, the biosynthesis of NO was still unknown. In 1988, L-arginine was shown to be involved in NO synthesis by vascular endothelial cells. These cells were cultured in medium deficient in L-arginine for 24 hours prior to stimulation with bradykinin. After

stimulation, a decrease in NO production was observed in cells cultured in L-arginine-deficient medium compared to cells cultured in complete medium with L-arginine. The production of NO could be restored by addition of L-arginine to the deficient medium. However, D-arginine did not restore NO production, which showed specificity in the substrate needed for NO synthesis (Palmer et al., 1988b).

Based on this observation, several reactions could hypothetically generate NO from L-arginine. The first hypothesis proposes that L-arginine is deiminated to form citrulline and NH_3, which is oxidized to form NO (Hibbs et al., 1987).

$$\text{L-arginine} + H_2O \xrightarrow{\text{Deiminase}} \text{L-citrulline} + NH_3$$

$$NH_3 + 1\ \tfrac{1}{2}\ O_2 \xrightarrow{\text{Oxidase}} NO_2^- + H_2O + H^+$$

The second hypothesis was the formation of hydroxylamine from N-hydroxy-L-arginine, which is acted upon by catalase to release NO (DeMaster et al., 1989).

$$\text{L-arginine} \xrightarrow{\text{Oxidase}} N^G\text{-Hydroxy-L-arginine}$$

$$N^G\text{-Hydroxy-L-arginine} \rightarrow \text{L-Citrulline} + \text{Hydroxylamine}$$

$$\text{Hydroxylamine} \rightarrow \text{NO}$$

where N^G is the guanidino nitrogen

The third hypothesis involved the formation of NO from L-arginine in a two-step process (Marletta et al., 1988). First, L-arginine is oxygenated to N-hydroxy-L-arginine; then the C=N bond of N-hydroxy-L-arginine is cleaved to form NO and L-citrulline

(Leone et al., 1991; Stuehr et al., 1991; Marletta, 1993; Korth et al., 1994). Both steps in this process require NADPH and O_2 (Leone et al., 1991; Marletta, 1993).

$$\text{L-arginine} \rightarrow N^G\text{-Hydroxy-L-arginine}$$

$$N^G\text{-Hydroxy-L-arginine} \rightarrow \text{NO} + \text{Diimide ornithine}$$

$$\text{Diimide ornithine} + H_2O \rightarrow \text{L-citrulline}$$

This hypothesis was shown to be correct by mass spectrometric analysis. In these experiments an ^{15}N labeled L-arginine (L-guanidino-^{15}N-arginine) was added to porcine aortic endothelial cells stimulated with bradykinin. One of the resulting products was identified as ^{15}NO (Palmer et al., 1988a). The second line of evidence for the third reaction was shown by the conversion of [H^3]L-arginine to [H^3]L-citrulline (Moncada and Palmer, 1990). This evidence strongly suggests that NO and citrulline are products of the same enzymatic reaction (Moncada and Palmer, 1990).

1.1b NO Synthases

Currently, there are three known NOS isozymes: NOS I, NOS II, and NOS III. All three isoforms use L-arginine, molecular oxygen and NADPH as substrates. These enzymes contain a heme group and require tetrahydrobiopterin (BH_4), FMN, and FAD as cofactors for the reaction (Brendt and Snyder, 1990; Hevel et al., 1991; Schmidt et al., 1991; Stuehr et al., 1991). All three NOS enzymes are completely inhibited by N-monomethyl-L-arginine (L-NMMA) and other L-arginine analogs (Moncada et al., 1991b;

McCall and Vallance, 1992; Joly et al., 1994). These isozymes have been found in many different cell types.

NOS I is constitutively expressed and requires Ca^{2+} and calmodulin for activity. NOS I is present in the brain (Knowles et al., 1989; Schmidt et al., 1989; Brendt and Snyder, 1990; Rodrigo et al., 1994), spinal cord (Dun et al., 1992), sympathetic ganglia (Dun et al., 1993), peripheral nitrergic nerves (Hassall et al., 1992; Saffrey et al., 1992; Sheng et al., 1992), epithelial cells in the lung, stomach, and uterus (Schmidt et al., 1992), platelets (Radomski et al., 1990a,b; Muruganam and Mutus, 1994), macula densa cells (Schmidt et al., 1992b), adrenal glands (Dun et al., 1993), and pancreatic islet cells (Schmidt et al., 1992). NOS I is also called the neuronal NOS (nNOS) because it is mainly expressed in neuronal tissues.

NOS II is not constitutively expressed and does not require Ca^{2+} for activation. Its synthesis can be induced by lipopolysaccharide (LPS) and cytokines in endothelial cells (Knowles et al., 1990a; Rees et al., 1990), hepatocytes (Knowles et al, 1990a,b), macrophages (Curran et al. 1989; Hevel et al., 1991; Lyons et al., 1992; Xie et al., 1992), neutrophils (McCall et al., 1989; Wright et al., 1989), and smooth muscle cells (Wood et al., 1990). NOS II is commonly called the inducible NOS (iNOS) because the gene can be induced to high levels of expression. The inducible NOS II is Ca^{2+} independent, regulated on a transcriptional level, and is induced by a wide variety of cytokines such as tumor necrosis factor alpha (TNFα) (Spink et al., 1995), interferon γ (IFN-γ) (Xie et al., 1993), and interleukin 1 beta (IL-1β) (Kanno et al., 1994). NOS II produces large amounts of

NO and for a longer duration compared to NOS I and NOS III (Moncada et al., 1991b; Nathan and Hibbs, 1991).

Isoform NOS III is constitutively expressed and present only in endothelial cells (Pollock et al., 1991,1993; Lamas et al., 1992; Marsden et al., 1992; Nishida et al., 1992; Sessa et al., 1992); therefore it is called the endothelial constitutive NOS (ecNOS). It is dependent upon Ca^{2+} and calmodulin for activation (Brendt and Snyder, 1990; Brendt et al., 1992; Forstermann et al., 1990,1991; Mayer et al., 1991; Nakane et al., 1991; Pollock et al., 1991).

1.1c Functional role of NO

After production by the endothelial cell, NO diffuses across the cell membrane into adjacent smooth muscle cells. NO reacts with the heme group of guanylate cyclase (GC) in smooth muscle. This produces a change in conformation which activates GC and increases the cyclic guanylyl monophosphate (cGMP) concentration within smooth muscle cells. The increase in cGMP causes a decrease in Ca^{2+} concentration by inhibiting Ca^{2+} mobilization from intracellular Ca^{2+} stores (Moncada, 1991a). The decrease in Ca^{2+} prevents activation of calmodulin, which results in myosin kinase inactivation. Active myosin kinase phosphorylates myosin, which forms polymers with actin, initiating contraction of the smooth muscle. Thus, calmodulin inactivation leads to vasodilation by preventing myosin activation (Guyton, 1991). Therefore, the physiological effect of NO is to produce vasodilation.

1.1d Transcriptional Regulation of iNOS

Since iNOS is regulated on a transcriptional level, analysis of the promoter region will aid in the understanding of the regulation of the gene. The promoter region of rat iNOS is reported to be 5.2 kb of which 3.2 kb plays a direct role in transcriptional regulation of iNOS mRNA (Zhang et al., 1998). Within the 3.2 kb rat iNOS promoter, there are many elements homologous to transcription factor consensus sequences: twenty-nine IFN-γ response elements (γ-IFNRE), two IFN-γ activation sequence (GAS), two tumor necrosis factor response element (TNF-RE), two cAMP response elements (CRE), two nuclear factor kappa B (NF-KB) binding sites, and three activator protein 1 (AP-1) recognition elements (Zhang et al., 1998). These transcription factor elements identify potential trans-activator proteins that can bind and cause induction or repression of the gene.

iNOS regulation has been heavily investigated in macrophages and smooth muscle cells. In macrophages, an NF-KB consensus site at -83 was found to be important for LPS stimulation of murine iNOS transcription. Deletion of this NF-KB site led to a decrease in reporter gene expression in LPS-stimulated cells (Lowenstein et al., 1993; Xie et al., 1993, 1994). However, in the vascular smooth muscle cell line A7r5, the NF-KB site at –1000 in the rat iNOS promoter seems to be important for conferring transcriptional induction by LPS (Zhang et al., 1998). Deletion of the –1000 NF-KB site decreases reporter gene expression almost 10-fold. These data suggest that iNOS regulation by NF-KB could be tissue specific, i.e., the –83 NF-KB site is important for iNOS transcription in

macrophages where as the NF-KB site at –1000 is important for iNOS transcription in smooth muscle cells.

Perrella et al. (1996) also tested the significance of the NF-KB site in the iNOS promoter. A reporter vector containing the region -400 to -1 of the mouse iNOS promoter region was transfected into rat aortic smooth muscle cells. These cells were either treated with LPS or pre-treated with the NF-KB inhibitor pyrollidine dithiocarbamate (PDTC) followed by LPS treatment. Pre-treatment with PDTC did not decrease LPS stimulation which suggests that NF-KB is not required for LPS-mediated induction. These inconsistencies warrant further investigation to determine which transcription factor(s) are necessary for iNOS induction in endothelial cells.

1.2 Nuclear Factor-Kappa B and Activator Protein-1

The MnSOD and iNOS genes contain a large variety of transcription factor response elements in their 5' promoter regions. Comparison of their sequences revealed that both promoters of both genes contain NF-KB and AP-1 response elements.

1.2a Nuclear Factor Kappa B

NF-KB was first characterized as a nuclear protein which bound to a 10 base pair (bp) region (GGGYNNCCY) of the kappa light chain enhancer (Sen and Baltimore, 1986a,b). A direct correlation between kappa light chain expression and NF-KB activity initially suggested that NF-KB might be a critical regulator for mature B cells (Sen and

Baltimore, 1986b; Atchison and Perry, 1987; Leonardo et al., 1987). However, NF-KB is found in almost every cell type and can be activated by LPS and phorbol myristic acid (PMA) (Grilli et al., 1993; Baeuerle and Henkel, 1994). A large number of inducible genes contain NF-KB response elements, and NF-KB can bind to these elements within a few minutes of stimulation (Grille et al., 1993; Baeuerle and Henkel, 1994). These findings indicate that NF-KB is a transcription factor that plays a global role in gene regulation (Baeuerle and Henkel, 1994).

NF-KB was initially purified as a heterodimer composed of p50 and p65 subunits (Baeuerle and Henkel, 1994). Sequencing of the genes encoding these two proteins revealed that they are members of a much larger protein family called rel transcription factors (Bours et al., 1990; Ghosh et al., 1990; Kieran et al., 1990; Meyer et al., 1991; Nolan et al., 1991; Ruben et al., 1991; Blank et al., 1992). NF-KB is now described as a homodimer or heterodimer of rel family members. The rel protein family is divided into two groups based on structure, function, and synthesis (Thanos and Maniatis, 1995). The first group of rel proteins contains p50 (NF-KB1) and p52 (NF-KB2), which are processed forms of their precursors p105 and p100, respectively. The mature p50 and p52 have a nuclear translocation signal and rel homology domain which facilitates DNA binding and rel protein dimerization. These mature proteins form dimers with other rel proteins and the resulting dimers can translocate to the nucleus; their unprocessed precursors, p105 and p100, can also form dimers with rel proteins but cannot translocate into the nucleus because the unprocessed protein carboxy terminus masks the nuclear translocation signal (Beg et al., 1992; Blank et al., 1992; Hatada et al., 1992; Henkel et

al., 1992; Liou et al., 1992). The second group of rel family members consists of p65 (rel-A), rel-B, and c-rel. These rel family members encode rel homology domains and one or more transcriptional activation domains (Thanos and Maniatis, 1995).

All members of both rel groups can form homodimers or heterodimers with other rel family members (Thanos and Maniatis, 1995). It is currently believed that the selection of dimerization partners directs either activation or inactivation of a NF-KB complex. Thus, the classical p50/p65 heterodimer is transcriptionally active, as are the combinations of p50/c-rel, p65/c-rel, and the p65 homodimer. However, the p50 and p52 homodimers are inactive and can actually repress NF-KB dependent transcription (Ballard et al., 1992; Lernbecher et al., 1993; Brown et al., 1994). The discovery of new dimer combinations and activities can lead to a better understanding of NF-KB based transcriptional enhancement or repression.

NF-KB dimers are retained in the cytoplasm by binding to a third protein subunit called inhibitor kappa B (IKB). IKB sequesters NF-KB in the cytoplasm by masking the nuclear translocation signal on p50 (Baeuerle and Baltimore, 1988a,b). IKB is a member of a family of proteins that has the distinguishing presence of multiple conserved ankyrin repeats. It is believed that ankyrin repeats interact with the rel homology domain of NF-KB (Davis et al., 1991; Haskill et al., 1991; Franzoso et al., 1992; Inoue et al., 1992; Gilmore and Morin, 1993; Naumann et al., 1993). IKB must be removed from NF-KB to produce a nuclear transcription factor, which can bind DNA and enhance transcription. Currently, the IKB family consists of IKB-α, IKB-β, IKB-γ, and Bcl-3. All of the IKB proteins, except Bcl-3, inhibit NF-KB activity (Ohno et al., 1990; Davis et al., 1991;

Haskill et al., 1991; Tewari et al., 1992; Inoue et al., 1992; Liou et al., 1992; Bhatia et al., 1991; Franzoso et al., 1992; Hatada et al., 1992; Wulczyn et al., 1992).

1.2b Activation of NF-KB

Activation of NF-KB requires a series of events which allows it to enter the nucleus, bind DNA and induce transcription. The first step in NF-KB activation is the stimulation of the cell by one of a diverse group of stimulators, including LPS, IL-1, IFN-γ, TNF-α, and ultraviolet light. All of these stimulators initiate a pathway in which a protein kinase, inhibitor kappa B kinase (IKK), is activated and phosphorylates the IKB subunit (Chen et al., 1995). This phosphorylation targets IKB for ubiquitinization and initiates IKB degradation by the proteosome (Thanos and Maniatis, 1995). After IKB is degraded, the nuclear translocation signal of the NF-KB dimer is unmasked, which allows NF-KB to translocate into the nucleus, bind its consensus sequence, and enhance transcription of its target genes (Thanos and Maniatis, 1995). One of the important genes that NF-KB induces is IKB-α (de Martin et al 1993; Beg et al., 1993; Brown et al., 1993; Sun et al., 1993). Entry of NF-KB into the nucleus causes induction of IKB-α, which binds and inactivates NF-KB. This forms a negative feedback loop, in which NF-KB directly increases the production of its inhibitor and leads to its own inactivation (Kopp and Ghosh, 1995).

The activation cascade described above is typical for NF-KB; however, different stimulators can have specific effects on NF-KB activation. Stimulation of NF-KB by

TNF-α or PMA produces a rapid transient activation of NF-KB, whereas LPS and IL-1 produce a prolonged activation of NF-KB in a pre-B cell line. This difference in activation, termed the biphasic response, is based on partnering with different IKBs (Thompson et al., 1995). IKB-α is degraded by proteolysis when any NF-KB stimulator initiates activation of NF-KB. The degradation of IKB-α causes a transient activation of NF-KB. In contrast to IKB-α, IKB-β is only degraded when the cells are stimulated with IL-1 or LPS. After IKB-β is destroyed, activation of NF-KB persists for several hours (Thompson et al., 1995).

1.2c Activator Protein 1

AP-1 is a transcription factor that binds the DNA consensus sequence TGA(C/G)TCA (Foletta et al., 1998). AP-1 is comprised of multiple protein complexes from two protein families, jun and fos. The jun family of proteins includes c-jun, jun B, and jun D (Maki et al., 1987; Ryder et al., 1988, 1989; Hirai et al., 1989), and the fos family of proteins includes c-fos, fos related antigen 1 (Fra-1), Fra-2, and fos B (Cohen et al., 1989; Matsui et al., 1990; Nishina et al., 1990; Zerial et al., 1989). Members of these two families can form dimeric complexes and produce an active AP-1 transcription factor complex.

Dimerization between jun and fos is facilitated by a leucine zipper motif, which is present in both subunits. Leucine zippers are amphipathic α helices, in which leucine residues are present every five amino acids; two leucine zippers can associate in a coiled coil arrangement (Kerppola and Curran, 1991). Leucine zipper proteins form very specific

interactions due to the presence of specific charged amino acid residues in the leucine zipper domain (O'Shea et al., 1989, 1992). These charged domains prevent fos-fos homodimerization, but do not prevent jun-jun homodimerization. Jun-jun homodimers are not as stable as the jun-fos heterodimers, which produce the most stable AP-1 complex (Nakabeppu et al., 1988; Halazonetis et al., 1988; O'Shea et al., 1989; Rauscher et al., 1988).

A second α helix region contiguous with the leucine zipper domain is rich in basic amino acids and forms the DNA binding domain of AP-1. Dimerization of AP-1 proteins juxtaposes the two basic α helices, and each domain provides an equal contribution to site-specific DNA recognition and binding (Kerppola and Curran, 1991; Nakabeppu and Nathans, 1989; Glover and Harrison, 1995). Transcription factors that contain leucine zippers and basic DNA binding domains are called b-Zip proteins (Lee, 1992). b-Zip transcription factor families include CREB, C/EBP, TEF/DBP, Maf/Nrl, and NF-E2 (Landschulz et al., 1988; Hai et al., 1989; Williams et al., 1991; Drolet et al., 1991; Kataoka et al., 1994). There are many instances in which b-Zip family members interact with one another, where heterodimers from different b-Zip families form heterodimers (Hai and Curran, 1991; Ryseck and Bravo, 1991; Kataoka et al., 1994). AP-1 can also form complexes with non-b-Zip proteins, such as the glucocorticoid receptor, basic helix loop helix Zip proteins, nuclear factor of activated T cells, and NF-KB (Yang-Yen et al., 1990; Stein et al., 1993; Pognonec et al., 1997; Jain et al., 1992).

1.3 The Endothelium

The endothelium is the innermost layer of cells in the arteries and veins. This layer of cells plays a large role in vasoregulation by producing and modulating vasoactive factors. Bloodborne compounds like angiotensin and bradykinin are metabolized by angiotensin converting enzyme within the cell and adenine nucleotides are metabolized by ectoadenosine trisphosphatase on the endothelial cell surface (Jaffe, 1985; Vane et al., 1990; Ramos, 1992; Marin and Rodriquez-Martinez, 1995; Simonescu and Simonescu, 1986). The endothelium produces a large number of paracrine factors including the vasoconstrictor endothelin, platelet activating factor, endothelium derived hyperpolarizing factor, tissue plasminogen activator, interleukins, von Willebrand factor, prostacyclin, and EDRF (Hanahan, 1986; Vanhoutte et al., 1986; Marin and Sanches Ferrer, 1990; Vane et al., 1990; Rubanyi, 1991).

Endothelial cells are both a target and a site of production for free radicals through mitochondrial oxidation, cyclooxygenase, and NO formation. Endothelial cells have a lower antioxidant capacity compared to other cell types (Bishop et al., 1985). Free radicals produced by pathological processes such as diabetes, inflammation, and atherosclerosis target the endothelium by its proximity to the blood (Rubanyi, 1988; Salvemini and Botting, 1990; Ward, 1991; Kehrer, 1993; Ross, 1993; Tesfamariam, 1994; Yu, 1994).

The effect of free radicals on the endothelium ranges from cell killing by activated macrophages to slight changes in the vascular conductance (Marin and Rodriquez-Martinez, 1995). Superoxide anion (O_2^-), hydroxyl radical, and hydrogen peroxide can

alter vascular tone (Marin and Rodriquez-Martinez, 1995). These reactive oxygen species (ROS) can produce vasoregulation by acting upon the synthesis or biological activity of endothelial derived vasodilators, such as NO and prostacyclin (Rubanyi, 1988; Marin and Rodriquez-Martinez, 1995). Therefore, it is important for endothelial cells to control the level of ROS. One control mechanism is mediated by the superoxide dismutases (SOD).

1.4 Manganese Superoxide Dismutase

Every cell in the human body needs oxygen (O_2) to produce energy. O_2 is required as an electron acceptor in the electron transport chain. This series of reactions also produces the byproduct O_2^-, which is highly reactive and can lead to direct DNA damage, inactivation of proteins, or lipid peroxidation. In order to protect the cell against these harmful reactions, SODs catalyze the removal of O_2^- by the following reaction:

$$2H^+ + 2\ O_2^- \xrightarrow{SOD} H_2O_2 + O_2$$

The dismutation reaction can occur non-enzymatically; however, SODs catalyze this reaction $1x10^4$ times faster than the non-enzymatic dismutation of O_2^- (Fridovich, 1975).

Three isoforms of SOD are known to exist in eukaryotic cells: (1) The copper-zinc SOD (CuZnSOD) which is found in the cytoplasm, (2) the extracellular CuZnSOD (ECSOD), and (3) the mitochondrial manganese SOD (MnSOD). CuZnSOD is constitutively expressed, while the MnSOD is highly regulated by various mediators of inflammation (Wong and Goeddel, 1988; Visner et al., 1990; Dougall et al., 1991; Valentine et al., 1992; Czaja et al., 1994). MnSOD synthesis can be induced by

TNFα, interleukins 1 and 6 (IL-1 and IL-6), IFN-γ and LPS. The induction of MnSOD can be inhibited by actinomycin D, an inhibitor of transcription, which indicates that the induction is caused by an increase in the transcriptional rate (Wong and Goeddel, 1988; Visner et al., 1990; Borg et al., 1992; Valentine et al., 1992).

1.4a Functional Role of MnSOD

In studies of MnSOD deficient mice, MnSOD has been shown to play a critical role in cellular protection in a variety of tissues. These mice exhibit metabolic acidosis, severe cardiac myopathy and lipid accumulation in the liver and skeletal muscle. They have a decreased level of activity for the enzymes sensitive to the redox state of the cell: aconitase, succinate dehydrogenase, and cytochrome C oxidase. These mice die within ten days of birth (Li et al., 1995).

MnSOD overexpression in mice exhibits a protective role against hyperoxic injury. Mice overexpressing MnSOD live longer in 95% O_2 than their non-transgenic littermates (Wispe et al., 1992). This suggests that MnSOD prevents cellular damage by removing O_2^- and preventing O_2^- mediated apoptosis. MnSOD could also play a role in tumor growth, since overexpression of MnSOD suppresses the tumorgenicity of human melanoma cells (Church et al., 1993), breast cancer cells (Li et al., 1995), and glioma cells (Zhong et al., 1997).

MnSOD plays an important role in the regulation of vascular tone. When O_2^- is present at high levels it can react with NO to form $OONO^-$ (peroxynitrite).

$$O_2^- + NO \rightarrow OONO^-$$

This reaction prevents NO from activating guanylate cyclase and prevents smooth muscle relaxation (McIntyer et al., 1999). Removal of O_2^- (by MnSOD) blocks the peroxynitrite reaction and allows NO to initiate smooth muscle relaxation. Through all of these studies MnSOD has been associated with many different diseases including cancer, diabetes, neurodegenerative diseases and hypertension. The largest problem remains in understanding the precise regulation of this gene.

1.4b Regulation of MnSOD

MnSOD is highly regulated and its transcription can be induced by stimulators of the inflammation response. The MnSOD promoter is atypical because it does not contain a TATA box or CAAT box, the standard recognition sequences for the basal transcriptional apparatus. Because the molecular regulation of TATA-less and CAAT-less promoters is not fully understood, the transcriptional regulation of MnSOD is under intense investigation (Kuo et al. 1999).

The MnSOD promoter region is at least 2.5 kb in length (Ho et al., 1993), and contains transcription factor recognition elements for SP-1, AP-1, AP-2, and NF-KB (Xu et al. 1999). When porcine aortic endothelial cells are stimulated by the pro-inflammatory compounds TNF-α, IL-1, or LPS, the mRNA for MnSOD is increased 6-25 fold depending on the stimulator (Visner et al., 1991, 1992). This shows that MnSOD is transcriptionally induced by cytokines and stress inducing factors. However, it is not clear how these stimulators produce the increase in MnSOD mRNA.

To determine the role of NF-KB in MnSOD transcription, Bedoya et al. (1995) treated porcine endothelial cells with PDTC and measured the MnSOD mRNA abundance. They found that MnSOD mRNA abundance was not decreased by PDTC and concluded that MnSOD does not require NF-KB for transcriptional induction. The mRNA for c-fos was found to be elevated after PDTC treatment. The investigators hypothesize that AP-1 could be indirectly playing a role in MnSOD transcriptional regulation. However, the study never directly examined the effect of AP-1 perturbation on MnSOD production. Maehara et al. (1999) showed that deletion of either NF-KB or CEPB sites decreased reporter expression and concluded that both NF-KB and CEBP are required for MnSOD induction. Xu et al. (1999) supported the role of NF-KB in MnSOD regulation. They generated a series of nested deletions within the human MnSOD promoter and 3' intronic region and found that NF-KB sites in the 3' intronic sequence were necessary for MnSOD stimulation by TNF-α. There is a distinct possibility that MnSOD is differentially regulated in different cell types and tissues.

2.0 Hypothesis and Specific Aims

LPS induces the MnSOD and iNOS genes and the promoters of both genes have AP-1 and NF-KB transcription factor recognition sites. We hypothesize that these transcription factors are important for LPS-mediated induction of MnSOD and iNOS. In this study, LPS was used for stimulation of primary rat aortic endothelial cells (PRAEC) and SV40 transformed aortic rat endothelial cells (SVAREC), based on the evidence that LPS induces iNOS and MnSOD transcription and NF-KB and AP-1 activity. The overall goal of this study was to determine how LPS induces transcription of MnSOD and iNOS. The specific aims were to:

(1) Establish or acquire an endothelial cell line suitable for the monitoring of MnSOD and iNOS reporter gene expression.

(2) Confirm that NF-KB and AP-1 activities are induced by LPS in PRAEC and SVAREC cell lines by monitoring the expression of NF-KB and AP-1 reporter genes.

(3) Demonstrate that MnSOD and iNOS are induced by LPS in SVAREC cells by monitoring the expression of MnSOD and iNOS reporter genes.

(4) Determine the role of NF-KB and AP-1 on LPS-meditated MnSOD induction in SVAREC cells by deleting their binding sites from the MnSOD reporter gene and by treating with inhibitors of NF-KB and AP-1.

(5) Determine the role of NF-KB and AP-1 on LPS-mediated iNOS induction in SVAREC cells by creating serial deletions and treating with inhibitors of NF-KB and AP-1.

3.0 Materials and Methods

3.1 Reagents

Agarose, ampicillin, Tris-HCl, NaCl, von Willebrand Factor Antibody (vWF), FITC-labeled smooth muscle α-actin antibody, and Endothelium Basal Medium were obtained from Sigma-Aldrich Corp. (St. Louis, MO). Trizol, Bluo-gal, phosphate buffered saline, fetal bovine serum, RPMI 1640, DMEM, penicillin and streptomycin, serum free endothelial plating medium, serum free endothelial growth medium, LB medium, Lipofectin, and Opti-mem medium were obtained from Life Technologies (Rockville, MD). Luciferase reagent, reporter lysis buffer, Acc65 I, Nhe I, Xho I, Hind III, Sfi I, Spe I, Nru I, Sma I, mung bean nuclease, Wizard miniprep kits, and Wizard midiprep kits were obtained from Promega (Madison, WI). 6-well Falcon Primaria coated plates, Falcon T-75 flasks, Matrigel, Tween 20, positively charged nylon membranes, Whatman filter paper, and polypropylene tubes were obtained from Fisher (Pittsburgh, PA). Top 10 Ultra Competent E. coli and the pCR2.1 cloning vector were obtained from Invitrogen (Carlsbad, CA). Enhanced Chemiluminescence (ECL) kit and BCA Protein Assay kit were obtained from Amersham-Pharmacia (Piscataway, NJ). A Northernmax Kit was obtained from Ambion (Austin, TX). T4 DNA Ligase was obtained from New England Biolabs (Boston, MA).

3.2 Ligation of inserts into pGL vectors

All ligations followed the manufacturer's protocol for T4 DNA ligase. In general, pCR2.1 and pGL plasmids were digested with compatible enzymes. A 2 fold or 4 fold molar excess of insert DNA compared to reporter plasmid was used in the ligation reactions. The T4 DNA ligase reaction was allowed to proceed overnight at 14°C. Ligation products were transformed into competent bacteria as described below.

3.3 Bacterial Transformation

Chemically competent bacteria (e.g. Top 10 cells) were transformed using the manufacturer's suggested guidelines. In general, 100ng of plasmid DNA were added to 50µl competent bacteria. This mixture was incubated on ice for 30 minutes, at 42°C for 30 seconds, and returned to ice. 200µl SOC bacterial medium was added and bacteria were incubated at 37°C for 1 hour to allow expression of antibiotic resistance. Bacteria were plated out on LB plates with 75 µg/ml ampicillin and incubated overnight to allow colony formation.

3.4 Blue/White screening

Some cloning vectors (e.g. pCR2.1) contain a multiple cloning site (MCS) within a functional lacZ gene. When a fragment is inserted into the MCS, the lacZ gene is disrupted; transformants with this vector are unable to hydrolyze the chromogenic substrate Bluo-gal, yielding white colonies. If lacZ is not disrupted, Bluo-gal is

hydrolyzed and blue colonies are formed. White colonies, tentatively considered insert-positive, were plated on a second Bluo-gal plate to confirm the lacZ phenotype. Plasmid DNA was purified from confirmed white colonies using Wizard miniprep columns.

3.5 Construction of MnSOD reporter vectors

A 2.5 kb MnSOD promoter fragment was obtained by direct amplification of genomic DNA in PCR containing 20mM Tris-HCl (pH 8.4), 50mM KCl with 1.5mM $MgCl_2$, 3mM dNTPs, 300pM MNSODPR1 and MNSODPR2 primers (Table 1), 100ng genomic DNA, and 2.5 units Taq polymerase. This reaction was performed for 30 cycles using 95°C for denaturing, 52°C for annealing, and 72°C for extension. The PCR products were inserted into TOPO TA cloning vector pCR2.1 which has topoisomerase covalently linked to the site of insertion. These vectors rely on single A overhangs for cloning any PCR product and the resealing activity of topoisomerase for plasmid closing.

Products of ligation reactions were transformed into commercially competent Top 10 cells which were plated onto LB agar medium with 75 μg/ml ampicillin and 1.5μg/ml Bluo-gal. Plasmid DNA was isolated from white colonies and tested for the MnSOD insert by restriction digests and automated sequencing. Since the pCR2.1 insert did not have compatible ends with the pGL-Basic vector, new primers were created with compatible ends for Acc65 I (MNSODPRACC1, Table 1) and Nhe I (MNSODPRNHE2, Table 1). These were used to reamplify the 2.5 kb fragment which was ligated into the pCR2.1 vector. One clone, designated MnSOD TA, contained the correct sequence.

Primer Name	Sequence
MNSODPR1	ACCAATCCCCATATCCCCCAGAAA
MNSODPR2	AGCCGCCGCCGAGACCAACCAAAG
MNSODPRACC 1	GCTTCGTCGGGTACCAATCCCCATATCCCCCAGAAA
MNSODPRNHE 2	GCTTCGTGGGCTAGCCGCCGCCGAGACCAACCAAAG
INOSPR1	CTCAGCCACCCATCTCTCAC
INOSPR2	TGACAGTAGCCATCAGGTATTTA
ACCINOSPR1	GCTTGCTGCGGTACCCTCAGCCACCCACCATCTCTCAC
XHOINOSPR2	GCTTGCGTGGCTCGAGTGACAGTAGCCATCAGGTATTTA
ΔNFKBMNSOD1	AAGACCACTGGGGTCCCACTCAAATCTCGAGACAACGCAA
ΔNFKBMNSOD2	TTGCGTTGTCTCGAGATTTGAGTGGGACCCCAGTGGTCTT
ΔAPMNSOD1	TCACTCAGGCATAAATTAAGAAGGCCCCTGATTACGCCA
ΔAPMNSOD2	TGGCGTAATCAGGGGCCTTCTTAATTTATGCCTGAGTGA

Table 1. DNA sequences of PCR primers used in this study.

The MnSOD promoter was transferred from MnSOD TA to pGL-2 Basic by digesting both plasmids with Acc 65 I and Nhe I. The digests were mixed in a 4:1 ratio, MnSOD TA to pGL-Basic, and ligated using T4 DNA ligase according to manufacturer's directions. A pGL-2 plasmid containing the MnSOD promoter was identified by automated DNA sequencing and was designated WT MnSOD-Luc.

3.6 Deletion of NF-KB and AP-1 Sites in MnSOD-Luc reporters

Recombinant PCR (Higuchi, 1988; Fig.1) was used to introduce deletions of the NF-KB and AP-1 sites into the MnSOD-Luc reporter. First, two sets of PCR primers were designed with the following features. (1) The primer pairs amplify adjacent regions of the promoter and generate PCR products that would overlap at the site of the deletion. (2) The internal primers are complementary and composed of twenty base pair regions that flank the site to be deleted. (3) The external primers flank the entire region and have restriction sites to facilitate subcloning of the final PCR product. Primer pairs and sequences designed for generating the NF-KB and AP-1 deletions are shown in Table 1.

Overlapping promoter fragments were generated by PCR containing 400ng WT MnSOD-Luc plasmid, 100pM external primer, 100pM internal primer, 1 unit of Taq polymerase, 20mM Tris-HCl, pH 8.4, 50mM KCl. Products were amplified for 25 cycles where each cycle consisted of a one minute denaturation step at 94°C, a one minute annealing step at 52°C and a one minute extension step at 72°C. PCR product formation was optimized by varying the final concentration of $MgCl_2$ from 1-7mM. These primary

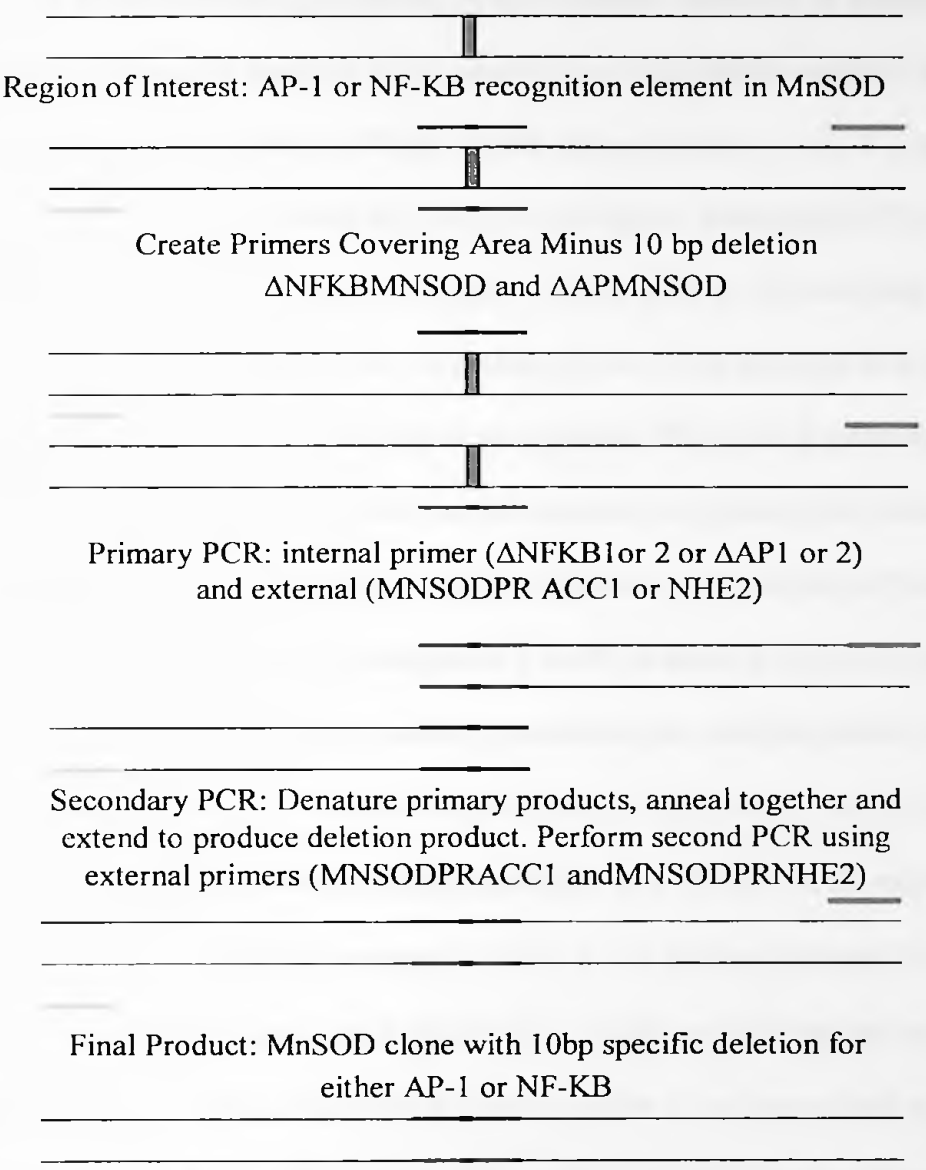


Figure 1. Recombinant PCR procedure for preparation of ΔNFKBMNSOD and ΔAPMNSOD promoters. In this study, ΔNFKBMNSOD and ΔAPMNSOD primers were used as the internal primers, and MNSODPRACC and MNSODPRNHE were used as the external primers.

PCR products were gel purified, mixed together, denatured by heating at 95°C for 10 minutes and used as template in the second stage PCR, which contained 4ul of primary PCR product reaction mixture, 100pM of both external primers, 1 unit Taq polymerase, 50mM Tris-HCl, pH 8.4, 50mM KCl, and 1.5mM $MgCl_2$. Secondary PCR products were amplified as described for primary PCR product amplification. The resulting 2.5 kb PCR product was subcloned into TOPO TA plasmid pCR2.1. The presence of the promoter insert was screened by restriction digestion of plasmid DNA from β-galactosidase negative (white) colonies. Verification of the MnSOD sequence and binding site deletions were confirmed by automated sequencing. pCR2.1 subclones, which contained the appropriate NF-KB or AP-1 deletions, were designated TA-ΔNF-KB and TA-ΔAP-1, respectively.

Luciferase reporters were made by transferring the deletion promoters from pCR2.1 subclones to pGL2-Basic as follows. Plasmid TA-ΔNFKB (or TA-ΔAP-1) and pGL2-Basic were digested with Acc65 I and Nhe I at 37°C for 1 hour. After restriction enzymes were inactivated by incubating at 67°C for 15 minutes, plasmid TA-ΔNFKB (or TA-ΔAP-1) and pGL2-Basic were mixed in a 4:1 molar ratio and ligated with T4 DNA ligase. The ligation mixture was transformed into TOP 10 competent cells which were then plated onto LB-amp plates. Ampicillin resistant transformants were screened for insert-positive pGL-Basic clones by PCR and restriction digests. The presence of the MnSOD promoter and appropriate deletions in NF-KB and AP-1 sites was confirmed by automated sequencing. These clones were designated ΔNF MnSOD-Luc and ΔAP MnSOD-Luc, respectively.

3.7 Cloning of iNOS Regulatory Region

A PCR based strategy was devised using primers INOSPR1 and INOSPR2 (Table 1) designed to amplify 1.7 kb (-1746 to –1) of the iNOS promoter. PCRs were performed using the standard PCR buffer (20mM Tris-HCl (pH 8.4), 50mM KCl, 100pM INOSPR1, 100pM INOSPR2, and 2.5 units of Taq) and a range of magnesium chloride concentrations (1.5mM, 2mM, 3mM, and 4mM). Two of these reactions (1.5 mM and 2mM $MgCl_2$) produced the predicted 1.7 kb fragment. This fragment was gel purified and digested with NcoI to initially confirm the product's identity. This digestion produced 1 kb and 700 bp fragments, the predicted products based upon the iNOS DNA sequence.

In order to facilitate insertion of the 1.7 kb fragment into pGL-3 Basic, the fragment was reamplified using upper and lower primers (INOSACC1 and INOSXHO2, Table 1) that contained Acc65 I and Xho I restriction sites compatible with the pGL-3 Basic polylinker. This PCR product was ligated into the TA TOPO 2.1 vector. The presence of the iNOS promoter was confirmed by restriction digest and automated sequencing (148 consecutive bases matched the published iNOS sequence) (Zhang et al., 1998). This clone was designated 1.7 INOS TA.

3.8 Construction of 1.7 iNOS-Luc

This 1.7 iNOS promoter fragment was inserted into the pGL-3 Basic to form expression vector 1.7 iNOS-Luc in the following manner. Plasmids 1.7 INOS TA and pGL-3 Basic were digested with Acc65 I and Xho I. The ligation reaction included a four-fold molar excess of iNOS-TA clone to pGL-3 Basic vector. Insert positive clones were

detected by amplification of 1.7 kb fragment with INOSACC1 and INOSXHO2 primers (Table 1) and confirmed by automated sequencing. One plasmid, iNOS1.7-Luc contained the correct iNOS sequence.

3.9 Construction of iNOS-Luc Deletion Series

Plasmid WT iNOS-Luc, which contains the 3.2 kb rat iNOS promoter inserted in pGL3-Basic, was received as a gracious gift from Dr. Hanfang Zhang (University of Georgia) and used to generate two nested deletions. Deletions were made by digesting WT iNOS-Luc with two restriction enzymes which cut only in the promoter and religating the plasmid backbone. The first deletion was made by cutting with Sma I and Spe I, which deleted 2.5 kb from –3200 to –680. Spe I overhangs were removed by treatment with Mung Bean nuclease (NEB, Boston, Mass). This blunt-ended plasmid was ligated back together with T4 DNA Ligase to form 0.7 iNOS-Luc. For the second deletion WT iNOS-Luc was cut with Sma I and Nru I, which deleted from –3200 to –266. This blunt ended plasmid was ligated together with T4 DNA Ligase to produce the 0.3 iNOS-Luc. The structures of both clones were confirmed by automated sequencing.

3.10 Transient Transfections

SV40 transformed rat aortic endothelial cells (SVAREC) and primary rat aortic endothelial cells (PRAEC) were transfected using the lipofectin reagent (Life Technologies, Rockville, MD). For each transfection, two solutions were prepared. (1)

5μl of lipofectin was diluted in 100μl of Opti-mem medium. (2) 1.5μg of luciferase reporter, 1μg of β-GAL reporter, and either 0.5μg of pGL-Basic or A-Fos were incubated in 100μl Opti-mem for 15 minutes. Both of these solutions were combined and incubated for 45 minutes to allow the liposome-DNA vesicles to form. Vesicles were then diluted to a final volume of 2 ml with Opti-mem medium and overlaid onto cells (200,000 cells per well). After incubation for 19 hours at 37°C, the vesicle solution was replaced with fresh complete growth medium (DMEM+10%FBS). Incubation at 37°C was continued for an additional 48 hours, at which time cells were left untreated, treated with parthenolide, LPS, or a combination of both for 4 hours. The cells were then ready to assay for luciferase and β-galactosidase activity.

3.11 Establishment of primary endothelial cell cultures

Rat aortic endothelial cell cultures were prepared by the method of McGuire and Orkin (1987) with some modifications. Thoracic aortae were removed from male Sprague Dawley rats at 9 weeks of age under aseptic conditions and placed in RPMI-1640 plus 20% serum. A longitudinal dissection of each aorta produced a sheet of aorta with the endothelial side of the vessel facing up. The aortic sheet was then cut into two halves that remain connected at the top; this allowed for the determination of the orientation of the endothelium. Each aorta half was then cut into approximately 15 2mm^2 aortic segments. Each segment was placed endothelial side down into the well of a 12 well plate coated with 35μl of matrigel, supplemented with 50μl complete medium (RPMI 1640 plus 20%

fetal bovine serum, 100μg/ml penicillin and 100μg/ml streptomycin), and incubated at 37°C in 5% CO_2 for 24 hours. At this point, an additional 50μl complete medium was added to each segment and incubation was continued for an additional 96-144 hours. The aortic segments were then removed and 250μl complete medium was added to allow attached cells to grow. Cell growth occurred mainly in the area peripheral to the original position of the aortic segment and was continued until 70% confluence was reached. During this period, complete medium was added every three to four days. In some cases, addition of medium would cause segments to lose attachment to the matrigel and float in the medium. This floating prevented cell growth in those specific wells and decreased the yield of endothelial cells.

Confluent endothelial cells have a cobblestone appearance which is characterized by the presence of triangular or rectangular cells that are somewhat rounded compact and placed side by side (similar to a cobblestone street) (McGuire and Orkin, 1987) (Fig. 2). Because all of the initial primary endothelial cell cultures failed to maintain this appearance, it was necessary to optimize pure endothelial culture production.

Optimization began by testing four different media with the same explant technique. These media were RPMI-1640 with 20% serum, RPMI-1640 with 3% serum, serum free endothelial plating medium, and endothelial basal medium with 0.5% serum (EBM). All four media supported initial growth. However, only RPMI-1640 with 20% serum and EBM supported cell growth after aortic segment removal. EBM cultures were confluent seven days after segment removal, compared to ten days with RPMI-1640.

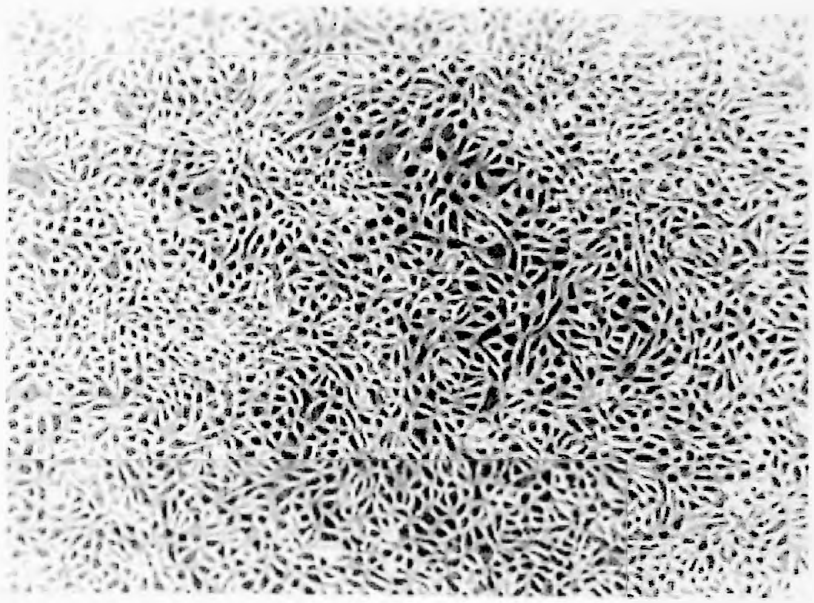


Figure 2. Morphology of primary endothelial cells. Note the rounded appearance of the cells and the contact inhibition.

Since EBM permitted more rapid cellular growth from the explant, this medium was used for the establishment of primary cell cultures.

When explant cells were incubated in EBM at 37°C under 5% CO_2, the cells grew more slowly after the first passage from 24 well plates to 6 well plates. This reduction in growth rate may have resulted from dilution of an endogenous growth factor(s) by the relatively large volume of medium. To correct for this dilution effect, cells were split into 12 well plates from the initial 24 well plate. In this modification, cell cultures were confluent after two weeks instead of four weeks.

A low percentage of explant cultures produced by this modified protocol exhibited growth and cobblestone morphology after three passages. In order to increase explant efficiency, two conditions (incubation time and medium volume) were tested. In the original protocol, aortic segments were incubated at least 96 hours before removal. Since it was possible that other cells, like smooth muscle and fibroblasts, could attach and grow on the matrigel, shorter incubation periods, 48 hours and 72 hours, were tested. In addition, decreased medium volume (10µl instead of 50µl) was added directly on top of the aortic segment of each well to prevent the segment from floating off of the matrigel. All three time points had segments that produced cells which grew after removal of the aortic segment. The time of growth before splitting the cells from 24 well plates to 12 well plates was dependent on the time of explant incubation. The 48, 72, and 96 hour explant cultures needed 14, 9, and 6 days, respectively, after explant growth before splitting. This difference was probably due to the number of cells that were growing off the explant at the time of removal. A second difference between the cultures became evident in the third

passage when all the cells were passaged from 12 well plates onto 6 well plates. The cells that were attached for 96 hours showed irregular growth, characterized by long spindly cells that grew on top of one another. Most of the 72 hour cultures showed some of the same irregular growth as found in the 96 hour cultures, but a few had the normal endothelial morphology. The majority of the 48-hour cultures had normal endothelial cell morphology, and only a few wells showed this irregular growth as in the 96-hour cultures.

These findings led to the final optimized protocol in establishment of a primary endothelial cell culture. Aortic segments were placed on matrigel coated wells of a 24 well plate and incubated with 10 µl of EBM medium for 24 hours, which provided enough time for the aortic segment to attach to the matrigel. Aortic segments were incubated for 48 hours in the presence of 30µl of EBM medium. Segments were then removed and 500µl of EBM was added. These cultures were grown until confluent and passaged every 2 weeks. This optimized procedure provided cell cultures that were pure aortic endothelial cells, based on vWF expression and absence of smooth muscle α-actin staining.

3.12 Endothelial Cell Typing

3.12a Western Blot

Cell lysates were prepared by adding 500µl lysis buffer (50mM Tris Cl (pH 8.0), 150 mM NaCl, 100µg/ml PMSF, 1µg/ml aprotinin, 1% Triton X-100) to the first well of a six well plate and detaching cells with a scraper. This lysate was added to the second well of the same plate, followed by cell scraping. This process was repeated for each well of

cells, until the cells from all six wells (Approx. $6x10^6$ cells) were combined into 500µl lysis buffer. The lysate was transferred to a 1.5ml microcentrifuge tube and sonicated for 10 sec at 30 MHz. A 50µl aliquot was removed and assayed for protein content by the BCA protocol using the following reaction:

$$\text{Protein} + Cu^{+2} \xrightarrow{OH^-} \text{tetradentate-}Cu^{+1}$$

$$Cu^{+1} + \text{bicinchoninic acid (BCA)} \rightarrow \text{BCA-}Cu^{+1}\text{ complex (purple color)}$$

The remainder of each sample was frozen at -70°C until needed for electrophoresis. Total protein (100µg) from each sample was prepared for electrophoretic analysis by adding an equal volume of loading buffer (100 mM Tris Cl (pH 6.8), 4% SDS, 0.2% bromphenol blue, 20% glycerol, 5% β-mercaptoethanol). Protein samples were denatured by heating at 100°C for 5 minutes, loaded into a 12% denaturing SDS polyacrylamide gel (Sambrook, 1989a) and electrophoresed at 35V for 20hrs. Gels were then placed in transfer buffer (39 mM glycine, 48 mM Tris base, 0.037% SDS, 20 % Methanol) for 20 minutes. Two pieces of Whatman filter paper and one piece of nitrocellulose were cut to fit the gel and placed in transfer buffer for 10 minutes. Protein was transferred from the gel to nitrocellulose membrane by a semi-dry blotter (Fischer Biotech, FB-SBD-2020). The transfer apparatus was assembled as follows. One piece of Whatman paper is placed on the bottom followed by the gel, nitrocellulose membrane, and a second sheet of Whatman paper. A glass rod was rolled across each surface to remove air bubbles which could prevent transfer of the protein. A 35V electric field was applied across the blot for 90 minutes to transfer the protein to the nitrocellulose membrane. The nitrocellulose was removed and cut on the

right top corner in order to provide orientation of the blot. Prestained protein markers (Bio-Rad, Hercules, CA) were cut off the blot and saved to determine the molecular weight of blotted proteins.

The nitrocellulose membrane was blocked by incubating in 10% milk solution (4g Dry Milk dissolved in 40 ml PBS) for 1hr, washed with PBS-Tween (PBS + 0.1% Tween-20) three times for 15 min and then incubated with an anti-vWF antibody A8052 (diluted 1:1000 in PBS-Tween) by rocking for one hour at room temperature. The membrane was then washed with PBS-Tween three times at 15 min intervals. The secondary goat anti-rabbit horseradish peroxidase labeled antibody was diluted 1:1000 in 5% blocking solution (5g Milk dissolved in 100ml PBS-Tween), added to the membrane, and incubated for one hour at room temperature on a rocker. Unbound secondary antibody was removed by a series of three 15-minute washes with PBS-Tween. Bound antibody was detected by enhanced chemiluminescence (ECL) as described by Amersham (Piscataway, NJ). For subsequent blotting, antibody was removed from nitrocellulose by soaking the membrane in stripping buffer (75mM Tris-HCl, 2% SDS, .35% 2-mercaptoethanol) for 30 minutes at 50°C, and washing twice with PBS-Tween for 15 minutes. Stripped membranes were re-probed with the smooth muscle α-actin primary antibody (1:1000) and glyceraldehyde phosphate dehydrogenase (GAPDH) primary antibody (1:1000).

3.12b Confocal Microscopy

Cells were plated onto 22 by 22mm coverslips (Fisher, Pittsburgh, PA) and incubated for at least 24 hours in DMEM at 37°C to allow for cell attachment and

spreading. Cells were fixed and permeabilized by addition of ice-cold acetone for one minute and then washed three times in PBS containing 0.5% Tween-20 pH7.5 (PBS-T). Cells were incubated in blocking solution for 10 minutes, and stained with a FITC-labeled anti-alpha-actin (1:500 dilution) for 30 minutes. Stained cells were visualized by mounting on a Nikon Diaphot Microscope; confocal microscopy was performed with a Bio-Rad 1024 scanning system with a krypton/argon laser. Micrographs were collected as serial projections of z-plane image acquisitions and analyzed using Lasersharp and Confocal Assistant Software (Bio-Rad, Hercules, CA)

3.13 Luciferase Assay

Cells were prepared for luciferase and β-galactosidase (B-Gal) assays with Reporter Lysis Buffer (RLB; Promega, Madison, WI) which supports the activity of both enzymes. Growth medium was removed from each well and the cells were washed once with PBS. RLB (400μl) was added to each well of a 6 well plate and the cells were incubated for 15 minutes. Each well was scraped with a Costar cell scraper and the lysate was collected into a sterile 1.5ml microcentrifuge tube. Each lysate was then placed on dry ice in order to complete lysis of the remaining intact cells. For luciferase assays, 50μl of each lysate were collected in duplicate and placed in a polystyrene tube. For β-galactosidase assays, a 100μl aliquot of each sample was transferred to a well from a 96-well microtitre plate in duplicate.

In the presence of ATP, luciferase catalyzes the oxidative decarboxylation of luciferin with the production of AMP, CO_2, and light which is measured on a luminometer.

$$\text{Luciferin} + O_2 + Mg^{2+} + \text{ATP} \xrightarrow{\text{Luciferase}} \text{Oxyluciferin} + \text{AMP} + CO_2 + \text{PPi} + \text{Light}$$

Each luciferase reaction was initiated by adding 50µl of luciferase reagent to 50µl cell lysate; the reaction was immediately placed in the luminometer for measurement of light produced. Luciferase activity, measured in light units, was calculated according to the following equations:

$$cL = \text{Light Unit Value} - \text{Background}$$

$$rL = cL/BGAL$$

Corrected light units (cL) were obtained by subtracting the background light reading from a reaction containing luciferase reagent and lysate, but not luciferase. cL values were corrected for transfection efficiency by dividing cL by the B-GAL activity in mU. This calculation provides the relative light (rL) unit used to measure luciferase activity. Each sample was assayed in duplicate.

3.14 Beta Galactosidase Assay

β-galactosidase reactions were initiated by the addition of 100ul of 4mM chlorophenolred-beta-D-galactopyranoside (CPRG) in Z Buffer to 100µl crude extract. β-galactosidase (B-GAL) catalyzes the releases of chlorophenol red which absorbs strongly at 550nm.

$$\text{CPRG} \xrightarrow{\text{B-GAL}} \text{Galactose} + \text{Chlorophenol red}$$

Reactions were incubated at 37°C for 3 hours and the absorbance at 550nm was measured. A standard curve was prepared by plotting β-galactosidase in milli units (.1 to 6) against the absorbance at 550nm. Since LacZ is driven by a constitutive promoter, the level of β-galactosidase activity reflects the efficiency of transfection.

3.15 Statistical Analysis

All statistical comparisons were accomplished with the Student's t-test. A comparison was considered significant when the $P<.05$. All groups had an n=3, unless otherwise noted.

3.16 Northern Blot

3.16a Probe Production

A random priming synthesis (RPS) kit (Life Technologies, Rockville, MD) was used to make DNA probes from cDNAs of rat MnSOD, mouse iNOS, and human GAPDH. Double stranded DNA was converted to single strands by boiling for 5 minutes and then chilling on ice. Each RPS reaction contained 25ng single stranded cDNA, 60μg/ml random octamer primers, 50mM Tris acetate (pH 6.8), 2.5 units of Klenow fragment, 5mM magnesium acetate, 1mM dithiothreitol, 10μM dATP, 10μM dGTP, 10μM dTTP, and 50μCi α–P^{32} dCTP; reactions were incubated at 37°C for 10 minutes. In order to determine the amounts of dCTP incorporated, 1μl of each probe was spotted onto glass filters in duplicate. One of the filters was left at room temp; the other was

washed with 50ml of 10% trichloroacetic acid and 50 ml of 75% ethanol. Each filter was placed into a scintillation vial containing 10 ml Universol (Fisher, Pittsburgh PA). Isotope incorporation was measured in a Beckman LS 1801 scintillation counter with all channels open. The percentage of isotope incorporated into cDNA was determined by dividing the cpm from the washed filter by the cpm from the unwashed filter. A total of $1x10^6$ cpm/ml of labeled cDNA in hybridization solution was used to probe nylon membranes.

3.16b RNA Samples

All RNA samples were prepared using Trizol reagent (Life Technologies, Rockville, MD). SVAREC cells were grown on 6 well plates until confluence was reached. Medium was removed and replaced with 1ml Trizol. This solution was pipetted repeatedly to ensure cell lysis and transferred to a 1.5ml microcentrifuge tube. Chloroform (200µl) was added to each sample and mixed by shaking for 15 seconds. Phases were separated by centrifugation at 12,000 x g for 15 minutes at 4°C. The aqueous phase, which contains the RNA, was collected and transferred to a new 1.5ml microcentrifuge tube. Two volumes of 95% ethanol were added, and samples were incubated at room temperature for 10 minutes. RNA precipitate was collected by centrifugation at 12,000 x g for 15 minutes at 4°C. After decanting the supernatant, the RNA was dried at room temperature for 10 minutes. RNA was dissolved in 50µl RNase free water and quantified by measuring the absorbance at 260nm. Amounts of RNA were calculated using the following formula:

$$\text{RNA } \mu g/ml = OD_{260}/ml \times 40\mu g/OD_{260} \times \text{dilution factor}$$

All RNA samples were stored at -70°C for later usage. All RNA samples were heated at 90°C for 10 minutes in Northern Max formaldehyde load buffer (Ambion, Austin, TX). 25μg RNA was separated by electrophoresis on a 1% agarose gel at 100V for 3 hours. RNA was transferred to positively charged nylon membranes using upward capillary transfer as described in Sambrook et al. (1985b).

3.16c Hybridization Conditions

Blotted membranes were incubated in 20ml UltraHyb solution (Ambion, Austin, TX) for 30 minutes at 42°C. ^{32}P-labeled DNA probes (1,000,000 cpm/ml) was added to the UltraHyb solution and allowed to hybridize to bound RNA overnight at 42°C. The nylon membrane was then washed twice with a low stringency wash at room temperature for 5 minutes each and twice with a high stringency wash at 42°C for 10 minutes each.

3.16d Autoradiography

Nylon membranes were wrapped in polyvinylchloride (Saran Wrap) and placed in a film cassette with two intensifying screens. Under a dark room safety light, a piece of Kodak X-ray film was placed over top of the membrane, with the RNA side of the membrane facing the film. The cassette was then closed and placed at -70°C for one to three days. After this exposure was complete, the film was developed in an automated developer (Alphatek, AX390SE).

4.0 Results

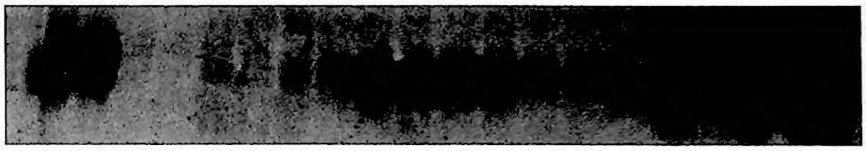
4.1 Endothelial Cell Typing

Primary endothelial cells are difficult to isolate as pure cultures from vascular tissue for several reasons. (1) The endothelium is a very thin layer of cells and is easily displaced by gentle agitation. Surgical removal of the aorta must be accomplished with precision and gentle handling in order to minimize loss of endothelial cells. (2) Removal of the endothelium exposes smooth muscle which is a significant source of cellular contamination. A number of commercially available endothelial cell cultures were either contaminated or not authentic endothelial cells (e.g. ECV304, an ATCC cell line). In order to insure that the primary endothelial lines were composed only of endothelial cells, cultures were tested for two markers, vWF which is found in endothelial cells and platelets but not smooth muscle cells, and α-actin which is present in smooth muscle cells but not endothelial cells. Western blotting was used to detect both marker proteins in PRAEC cells and SVAREC cells.

4.1a PRAEC Cell Typing

48 PRAEC cell cultures were prepared by the optimized isolation method; two cultures were prepared by the method described by Maguire and Orkin (1985). vWF protein was detected by western blot in 48 out of 48 PRAEC cell cultures prepared by the optimized method (Fig. 3, lanes 3-20 and data not shown) and the two cultures prepared

A. 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20



B. 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Figure 3. Detection of vWF and α-actin in PRAEC cells. Western blotting was used to measure the abundance of vWF and α-actin in PRAEC cells. Total cell protein (100μg) was separated by denaturing gel electrophoresis, transferred to a nitrocellulose membrane, detected with either an anti-vWF (panel A) or anti-α-actin (panel B) antibody and visualized using ECL. Lanes 1-2 are the initial cultures prepared with the original McGuire and Orkin (1987) protocol; lanes 3-20 are PRAEC cultures created using the optimized isolation protocol.

by the original protocol (Fig. 3. lanes 1 and 2). This finding suggested that the primary cell cultures contained endothelial cells, but did not determine the extent of possible contamination with smooth muscle cells. To address this question, the same membrane containing these 48 samples, was assayed for smooth muscle α-actin by western blot. The only two cultures positive for α-actin were those prepared by the original protocol (Fig. 3B. lanes 1 and 2). This evidence implies that the optimized explant protocol yields pure endothelial cell cultures.

4.1b SVAREC Cell Typing

In order to confirm the identity of the SVAREC cell line, four passages (#48, #50, #52, and #56) were tested for the presence of vWF and α-actin. All four passages were positive for vWF (Fig.4A lanes 1-4 and 6-9). Since the smooth muscle cell line, A7r5, which was intended as a negative control, actually produced vWF (Fig. 4A lanes 5 and 10), an osteoblastic cell line, URM-108, was used as a negative control for western blotting. vWF was not detected in this extract (Fig. 5, lane 3). This data demonstrates that the anti-vWF antibody exhibits specificity for its target and suggests SVAREC cells express vWF and are endothelial in nature.

To determine if SVAREC cells contained any smooth muscle cells, cell extracts were assayed for α-actin by western blotting. As expected, A7r5 smooth muscle cells were positive for α-actin (Fig. 4B, lane 5 and 10). However, all four SVAREC cell passages were also positive for smooth muscle α-actin (Fig. 4B, lanes 1-4 and 6-9). This

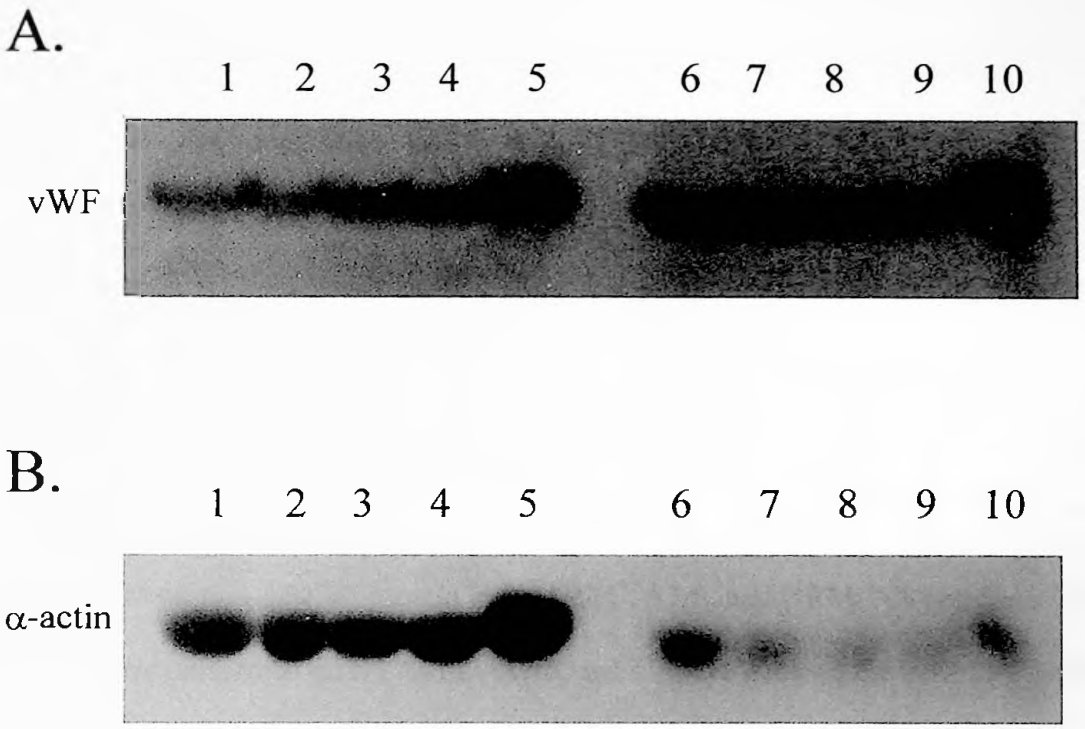


Figure 4. Detection of vWF and α-actin in SVAREC cells. Western blotting was used to measure the abundance of vWF and α-actin in SVAREC cells. Total cell protein [100μg (Lanes 1-5) or 50μg (Lanes 6-10)] was separated by denaturing gel electrophoresis. Proteins were transferred to a nitrocellulose membrane and detected with either an anti-vWF (A) or anti-α-actin (B) antibody. Lanes 1-4 and 6-9 are SVAREC cells lysates from passages 48, 50, 52, and 56, respectively. Lanes 5 and 10 are A7r5 cell lysates.

Figure 5. Detection of vWF protein in SVAREC, A7r5, and URM-108 cell lines. Western blotting was performed on SVAREC, A7r5, and URM-108 using the anti-vWF antibody. 50μg of total protein was separated by electrophoresis and transferred to nitrocellulose. This blot was incubated with an anti-vWF antibody and detected using ECL.

finding suggests that the SVAREC cell line could be contaminated with smooth muscle cells or that α-actin is present in an "atypical" form in SVAREC cells.

In order to distinguish between these two possibilities, SVAREC and A7r5 cells were stained with a FITC-labeled smooth muscle α-actin antibody and analyzed by confocal microscopy. Intertwined α-actin fibers typically found in smooth muscle cells were observed in A7r5 cells (Fig. 6D). In SVAREC cells, α-actin staining was only observed in the cell membranes (Fig. 6A and 6B). There was no natural fluorescence found in SVAREC cells left unstained (Fig. 6C). These observations suggest that SVAREC does not contain α-actin fibers, and that the α-actin antibody must recognize some other form of cellular actin. This finding implies that SVAREC cells are neither smooth muscle nor contaminated with smooth muscle cells.

4.2 Transfectability of PRAEC cells

In order to assess the ability of PRAEC cells to take up plasmid and express the luciferase reporter, PRAEC cells were transfected with two vectors: (1) pGL-2 Basic and (2) pGL-2 CNTR (Fig. 7). pGL-2 Basic carries a promoterless, enhancerless luciferase gene, and should produce low levels of luciferase when introduced into cells. pGL-2 CNTR contains a luciferase gene driven by an SV40 promoter and enhancer. When this construct is transfected into cells, it should produce a very high expression of luciferase in cells. Expression of pGL-2 CNTR plasmid was nearly five-fold higher than that of pGL-2

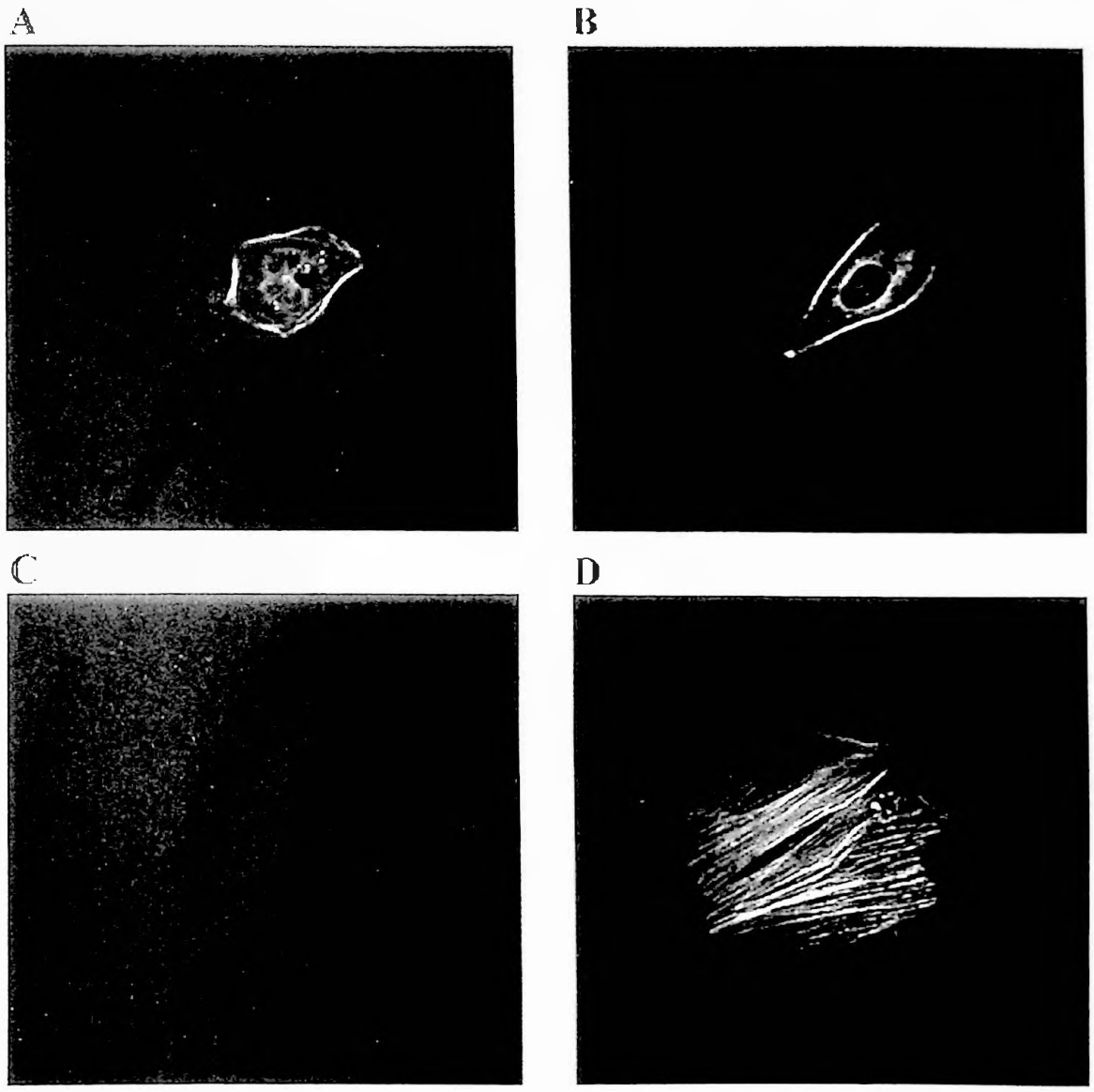


Figure 6. Detection of α-actin with confocal microscopy. SVAREC (panels A, B, and C) and A7r5 (panel D) cells were stained with a FITC labeled anti-α-actin antibody and visualized by confocal microscopy. Cells shown in panel C were not treated with antibody in order to assess background fluorescence of SVAREC cells.

A. pGL-2 Basic

B. pGL-2 Control

Figure 7. pGL Basic and pGL Control plasmids. The pGL Basic vector (A) contains no promoter or enhancer regions to initiate transcription of luciferase. The pGL Control vector (B) contains the SV40 promoter and enhancer.

Basic plasmid (Fig. 8). These findings showed evidence that PRAEC cells could be transfected and that luciferase is expressed.

4.3 AP-1 and NF-KB activity in PRAEC Cells

Since PRAEC cells could be transfected, luciferase reporters were acquired which measured the level of AP-1 and NF-KB transcription factor activity. The AP-1 Luc reporter (Fig. 9B) contains seven tandemly repeated AP-1 recognition elements 5' to the luciferase gene, while the NF-KB Luc reporter contains five tandemly repeated NF-KB recognition elements 5' to the Luc ORF (Fig. 9A). PRAEC cells were transfected with the NF-KB Luc reporter and either treated with LPS (100 μg/ml) or left untreated. LPS treatment increased NF-KB reporter expression by five-fold (Fig 10). PRAEC cells were also transfected with AP-1 Luc and either treated with LPS or left untreated. AP-1 activity increased upon LPS stimulation by 2.5-fold (Fig. 10). These data indicated that LPS stimulation of PRAEC cells activated both AP-1 and NF-KB.

4.4 MnSOD Regulation in PRAEC Cells:

Since LPS induced both NF-KB and AP-1 activity in PRAEC cells and since the recognition elements for both transcription factors are present in the MnSOD promoter, we asked if LPS also induces MnSOD expression. PRAEC cells were transfected with the WT MnSOD-Luc reporter and either stimulated by LPS or left

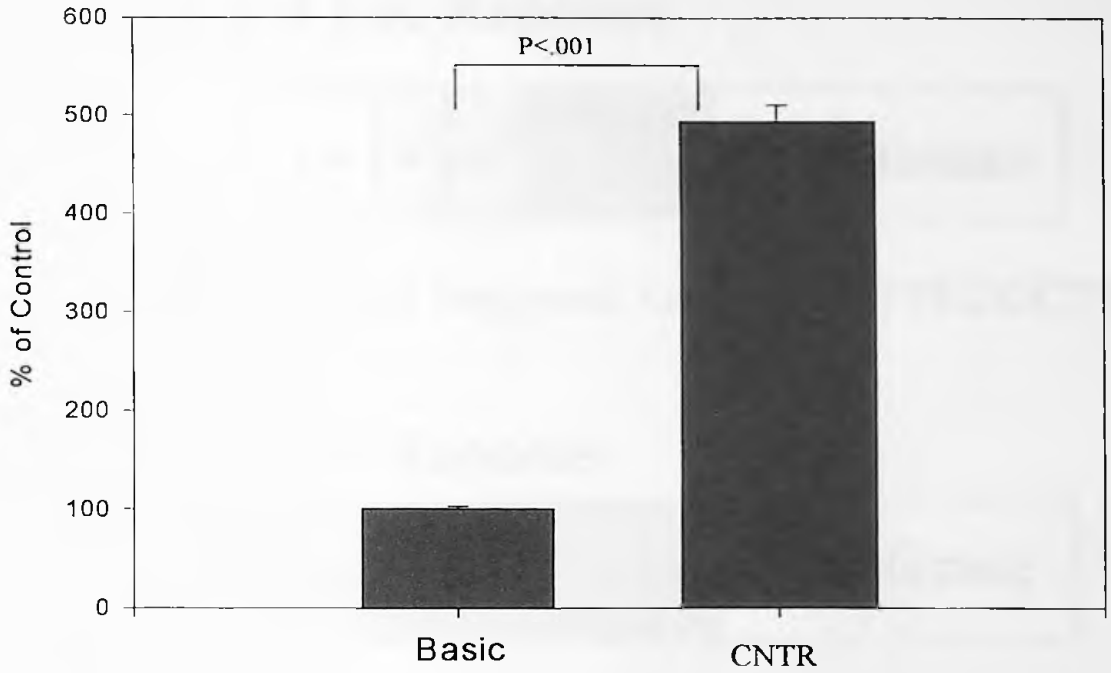


Figure 8. Expression of pGL-Basic and pGL-CNTR in PRAEC cells.

PRAEC cells were transfected with pGL-2 Basic and pGL-2 CNTR luciferase reporter plasmids. Luciferase activity was normalized to untreated controls for pGL-2 Basic transfected cells.

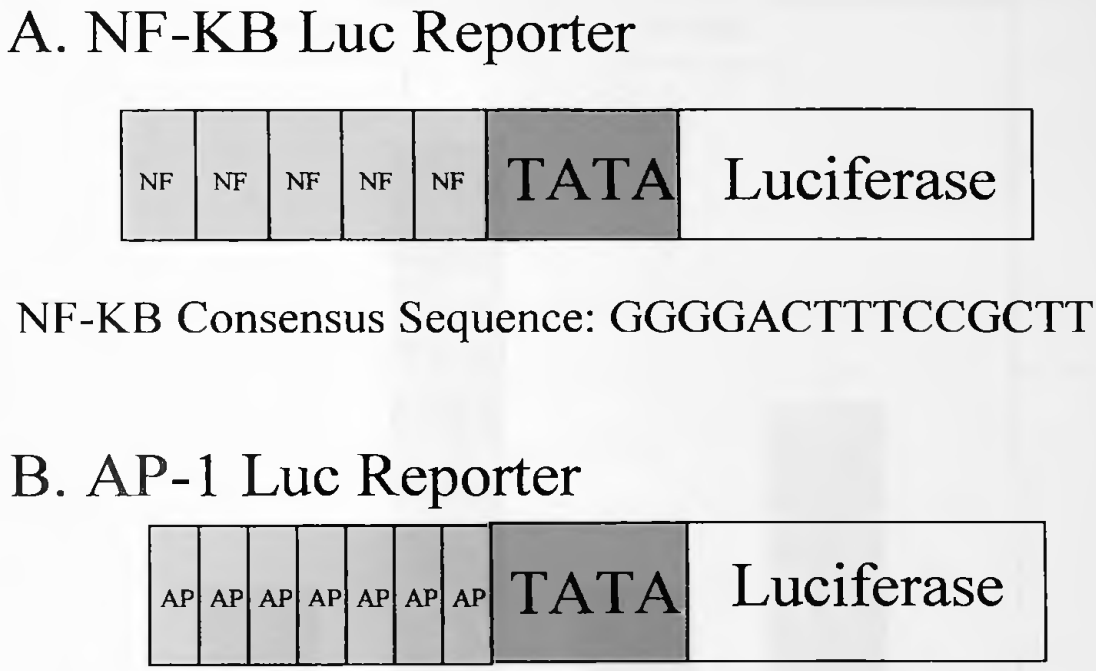


Fig 9. Schematic representation of NF-KB Luc and AP-1 Luc reporter genes. The NF-KB Luc vector contains five consensus sequences (NF) for NF-KB upstream of the TATA element. The AP-1 Luc vector contains seven AP-1 consensus sequences (AP) in front of the TATA promoter.

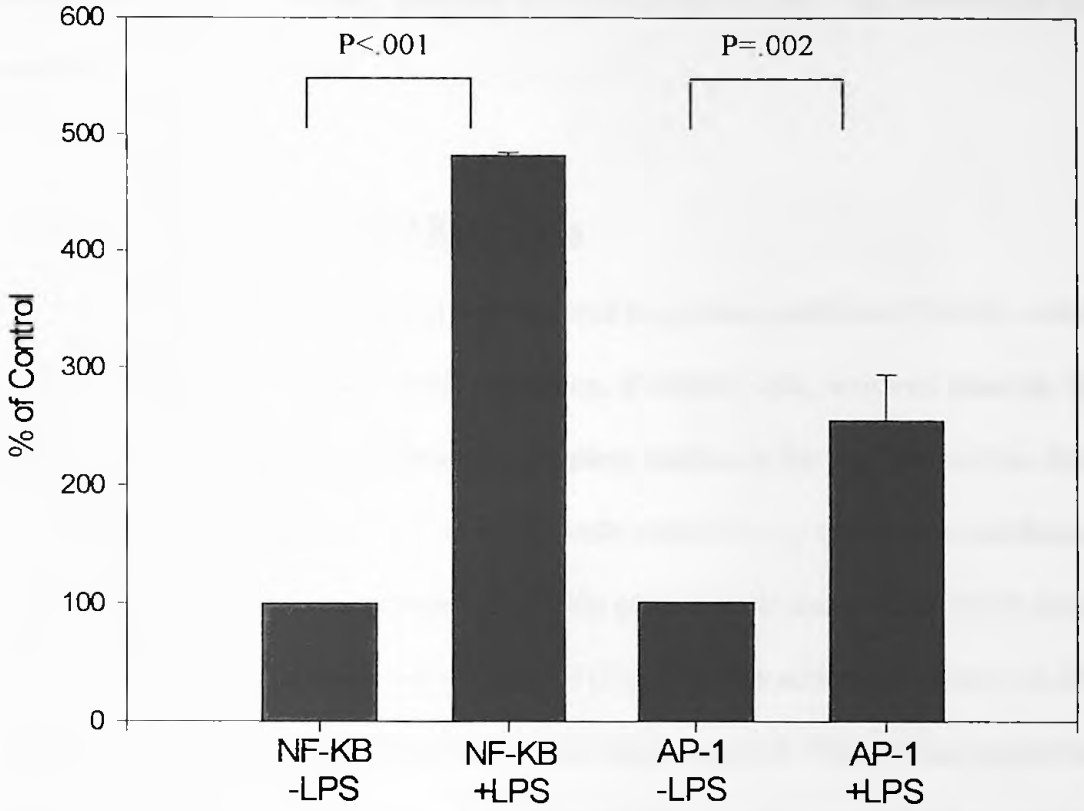


Figure 10. Expression of NF-KB and AP-1 reporters in PRAEC cells. PRAEC cells were transfected with NF-KB Luc and AP-1 Luc reporter plasmids and either treated with LPS (+LPS) or left untreated (-LPS). Luciferase activity was normalized to untreated controls for NF-KB Luc and AP-1 Luc transfected cells.

unstimulated (Fig.11). WT MnSOD-Luc was induced nearly three-fold by LPS treatment compared to unstimulated levels. This indicates that MnSOD transcription can be stimulated by LPS in PRAEC cells and that NF-KB and/or AP-1 may function as potential mediators of the response.

4.5 Transfectability of SVAREC Cells

A six-week growth period was required to generate sufficient PRAEC cells for a single transfection. Because of this restriction, SVAREC cells, acquired from Dr. Beatrice Chareau at INSERM, were selected to complete studies on the regulation of the iNOS and MnSOD genes. To determine if SVAREC cells could take up and express luciferase reporters, these cells were transfected with the pGL-2 Basic and pGL-2 CNTR vectors (Fig. 7) and luciferase levels were measured (Fig. 12). The activity of the pGL-2 CNTR reporter was 22-fold greater than that of pGL-Basic reporter. This finding demonstrates that SVAREC cells were able to take up plasmid DNA and express high levels of the luciferase reporter gene.

4.6 NF-KB and AP-1 Activity in SVAREC Cells

Since SVAREC cells were able to take up plasmid and express luciferase, their ability to activate AP-1 and NF-KB upon LPS stimulation was tested. In order to determine the activity of these transcription factors, SVAREC cells were transiently transfected with NF-KB Luc and AP-1 Luc reporter plasmids (Fig. 9) and then treated

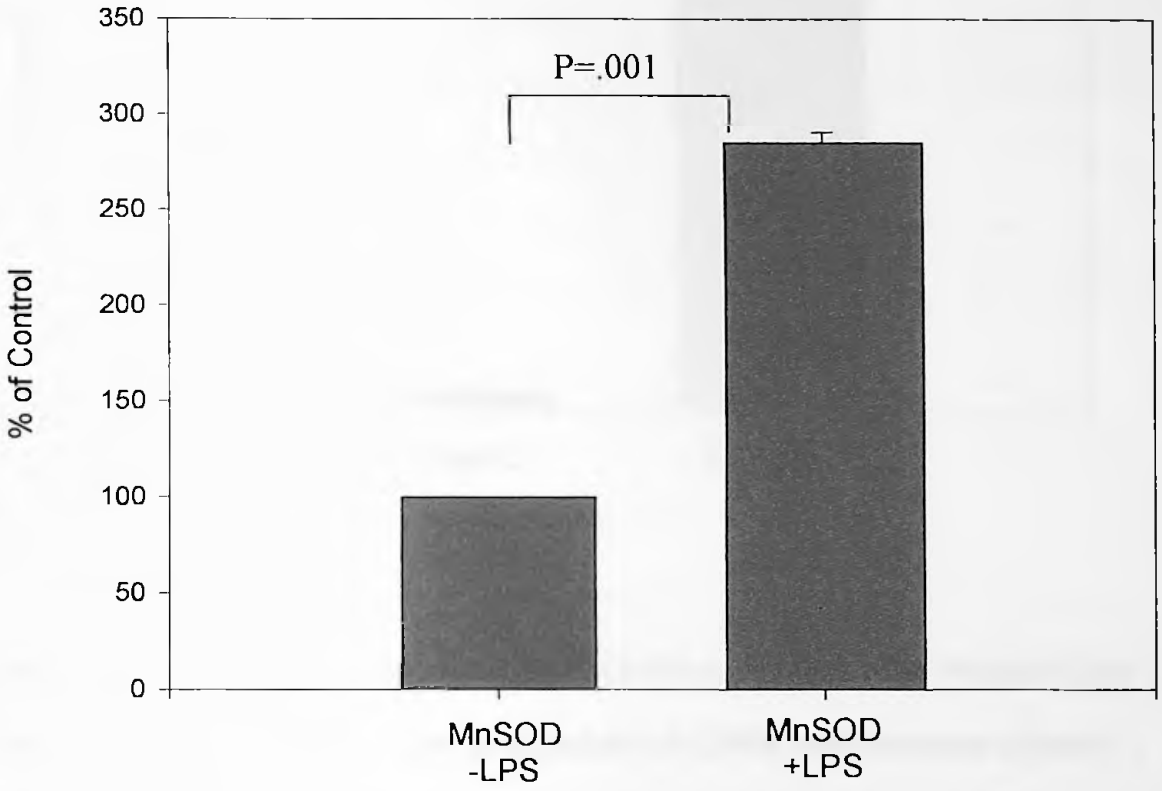


Figure 11. Expression of WT MnSOD-Luc reporter in PRAEC cells. PRAEC cells were transfected with WT MnSOD-Luc reporter and either treated with LPS (+LPS) or left untreated (-LPS). Luciferase activity was normalized to untreated controls for WT MnSOD-Luc transfected cells.

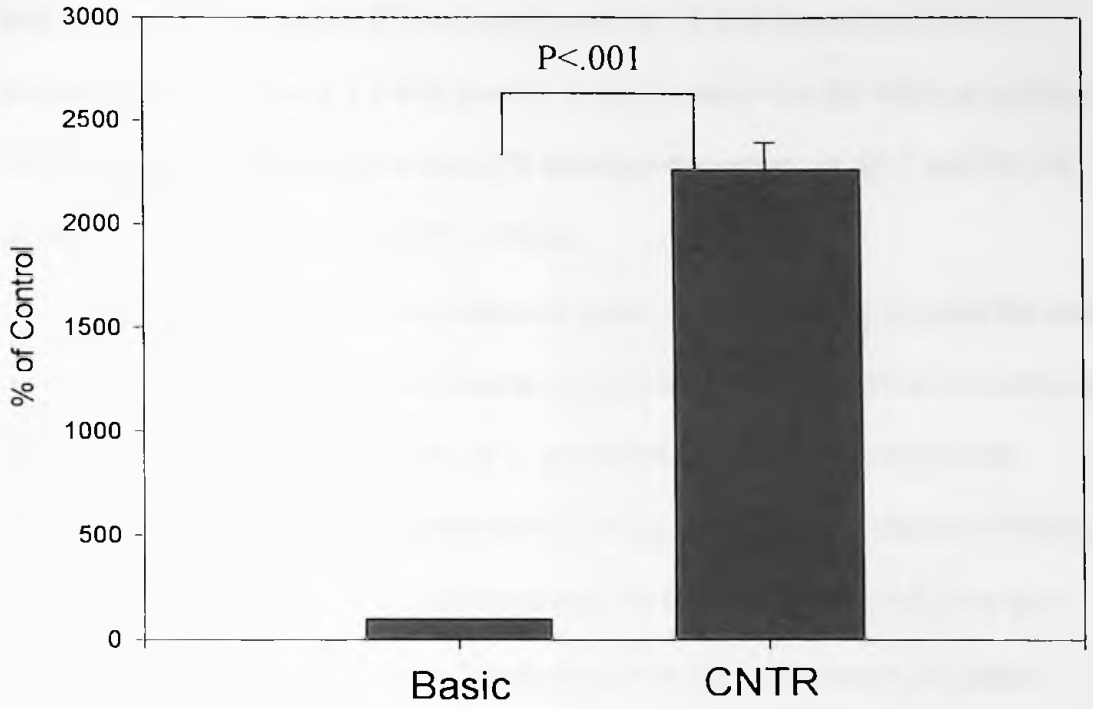


Figure 12. Expression of pGL-Basic and pGL-CNTR in SVAREC cells. SVAREC cells were transfected with pGL-2 Basic (n=6) and pGL-2 CNTR (n=6) luciferase reporter plasmids. Luciferase activity was normalized to untreated controls for pGL-2 Basic transfected cells.

with LPS or left untreated. LPS treatment caused a 2.2-fold increase in AP-1 Luc expression (Fig. 13) and a 2.5-fold increase in expression of the NF-KB Luc reporter (Fig. 14). These two findings suggest that LPS increases the activity of AP-1 and NF-KB transcription factors in the SVAREC cell line.

In order to confirm that the reporter genes were responding in a specific manner to increases in transcription factor activity, transfected SVAREC cells were treated with biological inhibitors of NF-KB and AP-1, parthenolide and A-Fos, respectively. Parthenolide prevents the degradation of IKB and prevents translocation of NF-KB to the nucleus. When SVAREC cells transfected with the NF-KB Luc reporter were pre-incubated with 10μM parthenolide 30 minutes before LPS stimulation, luciferase expression decreased by 50% (Fig. 14, compare 2nd and 3rd columns). Inhibition of reporter expression confirms that the NF-KB reporter requires NF-KB for expression. Pre-incubation of SVAREC transfectants with 10μM parthenolide for 30 minutes did not prevent LPS stimulation of AP-1 Luc reporter expression (Fig. 13, compare 2nd and 3rd columns). This evidence shows that the AP-1 Luc reporter does not require NF-KB for expression.

A-Fos is a dominant negative protein that binds c-jun and c-fos, but lacks an activation domain. A-Fos complexes bind to AP-1 sites and prevent transcription of AP-1 stimulated genes. In order to assess the requirement for AP-1 in NF-KB and AP-1 reporter expression, SVAREC cells were co-transfected with an A-Fos expression plasmid and either AP-1 Luc or NF-KB Luc reporters and stimulated with LPS.

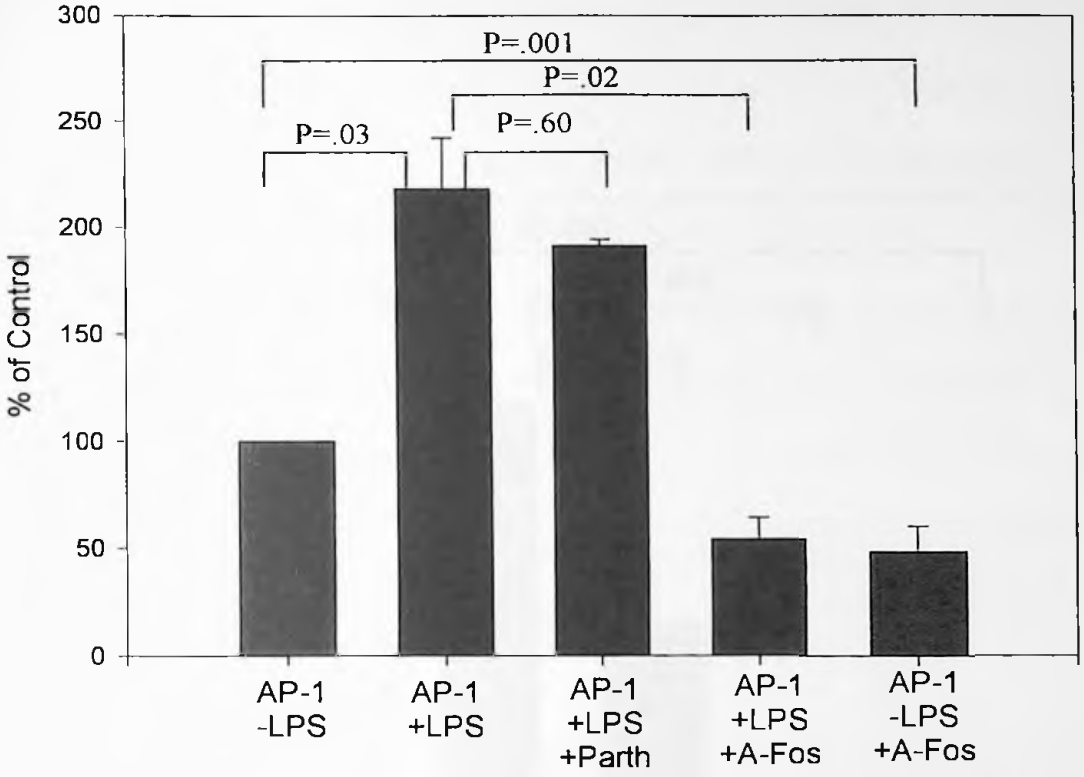


Figure 13. Expression of AP-1 Luc reporter in SVAREC cells. SVAREC cells were transfected with AP-1 Luc reporter plasmid and either treated with LPS (+LPS) or left untreated (-LPS). In addition to LPS treatment, some cultures were either treated with 10μM parthenolide (+Parth) or co-transfected with A-Fos (+A-Fos). Luciferase activity was normalized to untreated controls for AP-1 Luc transfected cells.

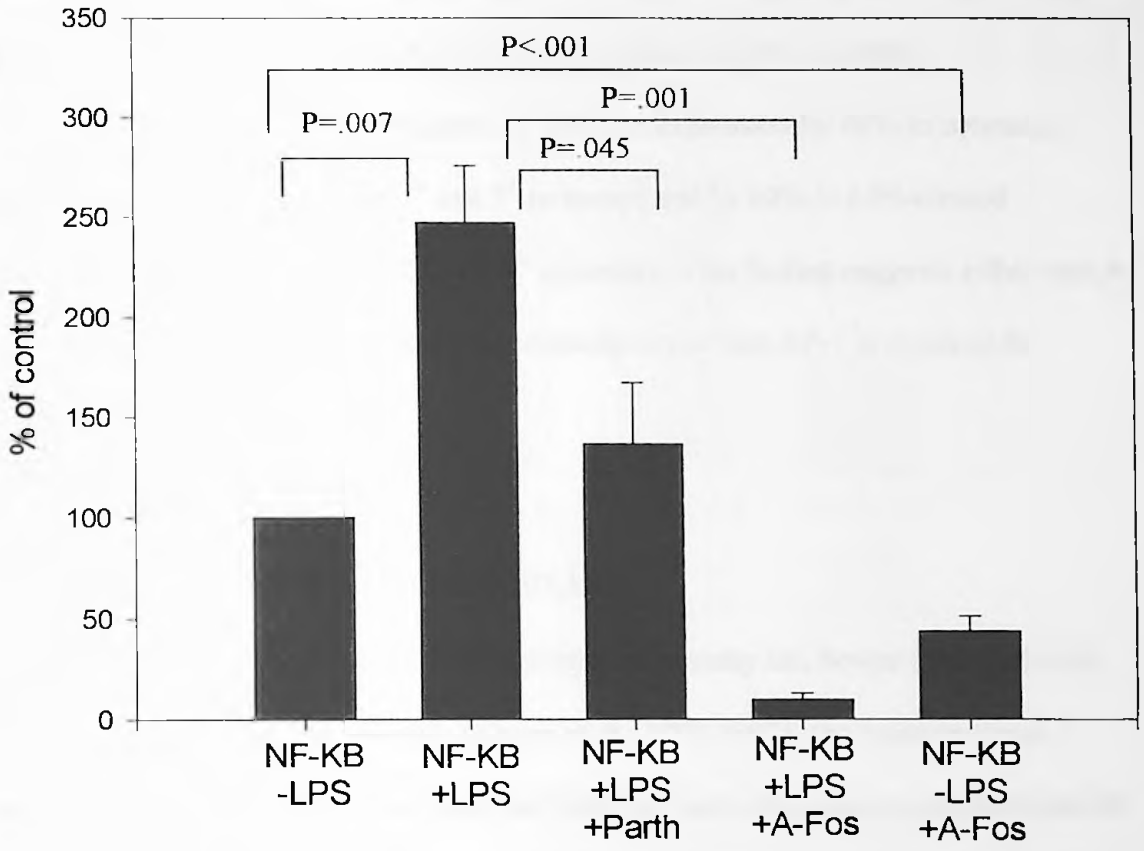


Figure 14. Expression of NF-KB Luc reporter in SVAREC cells. SVAREC cells were transfected with NF-KB Luc reporter plasmid, and either stimulated with LPS (+LPS) or left untreated. In addition to LPS treatment, some cells were either treated with 10μM parthenolide (+Parth) or co-transfected with A-Fos (+A-Fos). Luciferase activity was normalized to untreated controls for NF-KB Luc transfected cells.

Expression of A-Fos decreased AP-1 Luc expression by 50% in untreated transfectants (Fig. 13, compare 1st and 5th columns) and by 76% in LPS-treated transfectants (Fig. 13, compare 2nd and 4th columns). This evidence implies that the basal and induced expression of the AP-1 Luc reporter relies on AP-1 activity.

Expression of A-Fos decreased NF-KB Luc expression by 60% in untreated transfectants (Fig. 14, compare 1st and 5th columns) and by 90% in LPS-treated transfectants (Fig. 14, compare 2nd and 4th columns). This finding suggests either that A-Fos can prevent NF-KB from enhancing transcription or that AP-1 is required for activation of NF-KB.

4.7 Induction of MnSOD Reporter by LPS

Since LPS induces MnSOD transcription in primary rat, bovine (Mitchell et al., 1996), and porcine endothelial cells (Visner et al., 1991,1992), the transcriptional response of MnSOD to LPS was tested in SVAREC cells. In order to monitor MnSOD expression, SVAREC cells were transfected with WT MnSOD-Luc reporter and either treated with LPS or left untreated. Treatment of SVAREC transfectants with LPS induced WT MnSOD-Luc reporter expression 1.75-fold compared to unstimulated levels (Fig. 15, compare 1st and 2nd columns). This finding suggests that MnSOD induction in SVAREC cells is comparable to other endothelial cells (Visner et al., 1991,1992; Mitchell et al., 1996). Therefore, SVAREC cells could be considered to be an appropriate system for endothelial study.

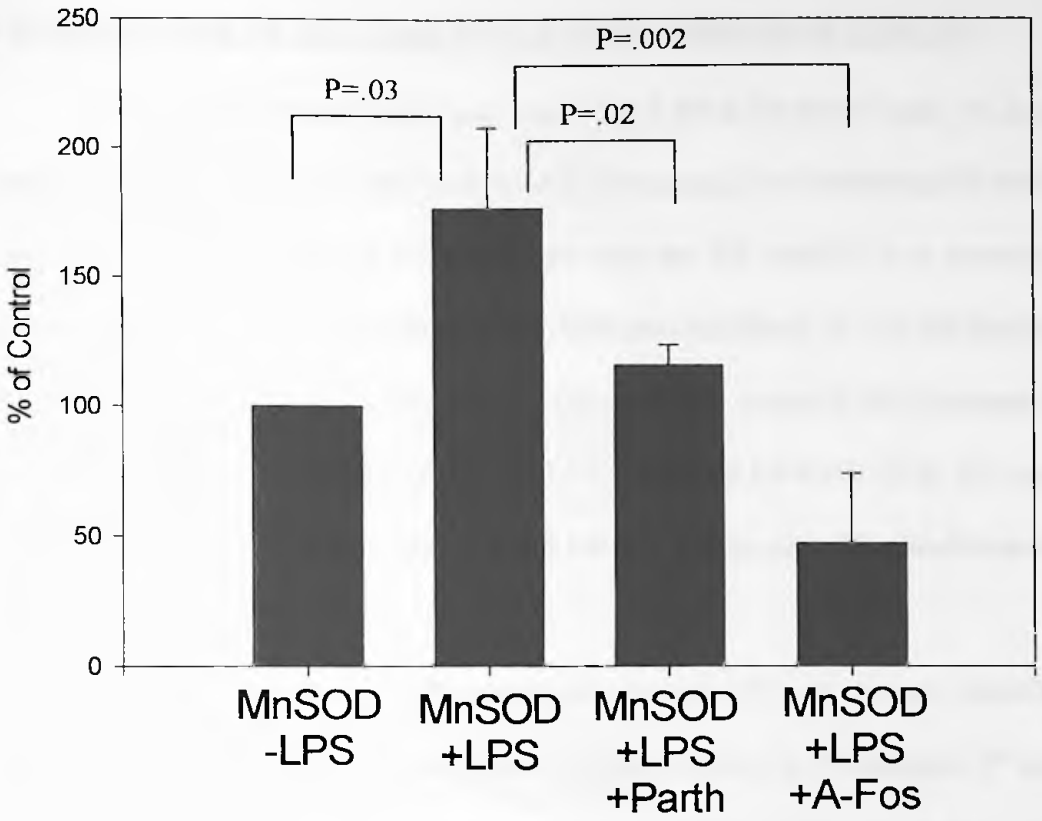


Figure 15. Expression of WT MnSOD-Luc in SVAREC cells. SVAREC cells were transfected with WT MnSOD Luc reporter plasmid, and the cells were either stimulated with LPS (+LPS) or left untreated (-LPS). In addition to LPS treatment, some cultures were either pre-treated with parthenolide (+Parth) or co-transfected with A-Fos (+A-Fos). Luciferase assays were normalized to untreated controls for WT MnSOD Luc transfected cells.

4.8 Requirement for AP-1 and NF-KB in the induction of MnSOD

Since MnSOD transcription was induced by LPS in SVAREC cells, we sought to determine the importance of AP-1 and NF-KB on transcriptional induction of MnSOD. This was tested by transfecting SVAREC cells with the WT MnSOD-Luc reporter and then treating transfected cells with LPS and biological inhibitors, A-Fos and parthenolide. In LPS-stimulated SVAREC cells, parthenolide treatment caused a 30% decrease in reporter expression compared to untreated LPS stimulated SVAREC (Fig. 15, compare 2nd and 3rd columns). This data suggests that NF-KB is important for stimulation of MnSOD by LPS.

Expression of A-Fos in LPS-treated cells caused a 71% decrease in MnSOD reporter expression compared to untreated SVAREC cells (Fig. 15,compare 2nd and 4th columns). However, because the A-Fos expression plasmid inhibited both NF-KB and AP-1 reporters, it is unclear if the action of A-Fos is against AP-1 or NF-KB or both.

4.9 Analysis of ΔNF MnSOD and ΔAP MnSOD in SVAREC Cells

In order to assess the role of NF-KB and AP-1 in MnSOD transcriptional regulation, MnSOD-Luc reporters were created with deletions in either the NF-KB site [ΔNF MnSOD-Luc, (-1443 to –1433)], or AP-1 site [ΔAP MnSOD-Luc, (-431 to –423)] (Fig. 16). SVAREC cells were transfected with WT MnSOD-Luc, ΔNF MnSOD-Luc, and ΔAP MnSOD-Luc reporters and then either treated with LPS or left untreated (Fig. 17). The WT MnSOD-Luc was induced 1.75-fold when treated with LPS; ΔAP

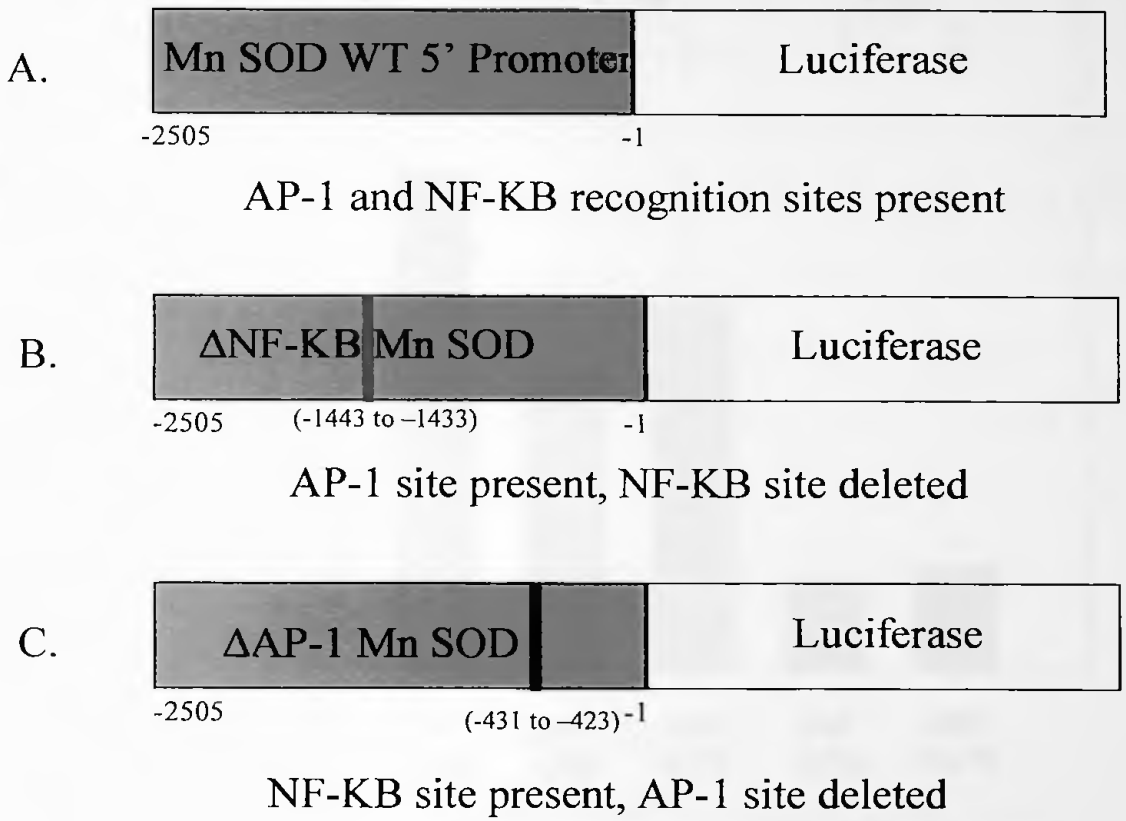


Figure 16. Schematic representation of the MnSOD-Luc reporter vectors. (A) WT MnSOD-Luc reporter which contains 2.5 kb of MnSOD promoter region 5' to the luciferase gene. (B) ΔNF-KB MnSOD-Luc reporter which contains the MnSOD promoter region with the NF-KB response element deleted. (C) ΔAP-1 MnSOD-Luc reporter which contains the MnSOD promoter region with the AP-1 response element deleted.

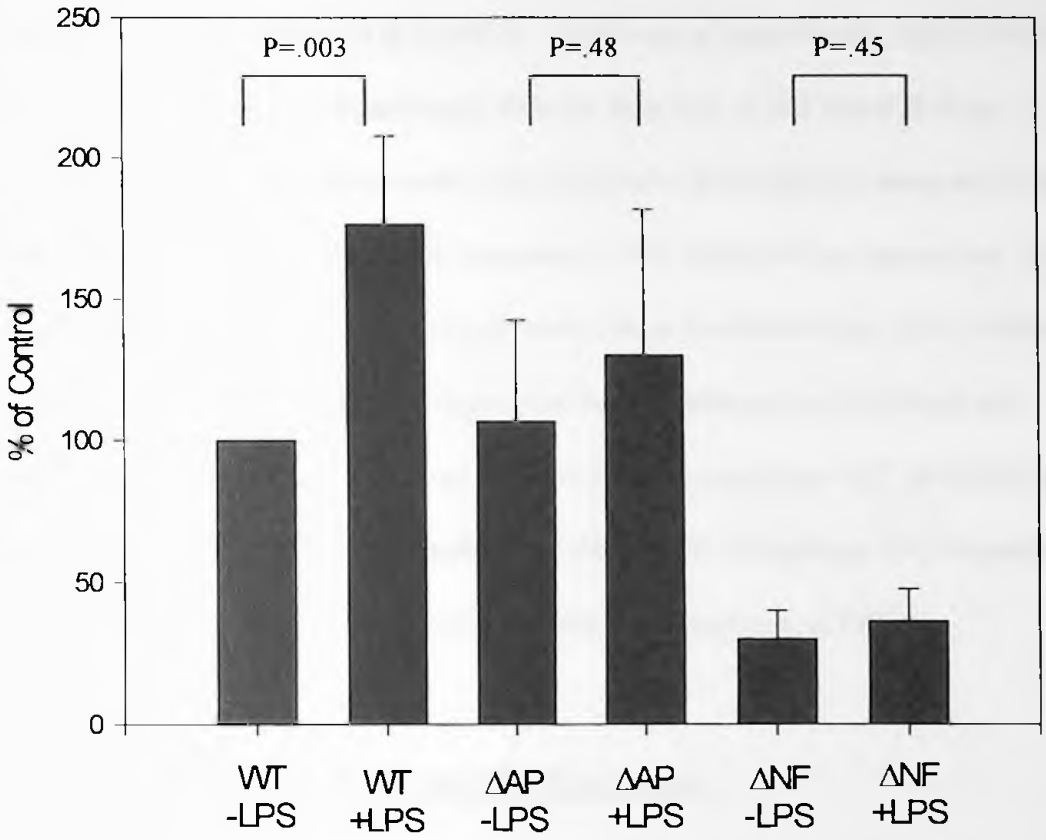


Figure 17. Effect of LPS in MnSOD reporter expression. SVAREC cells were transfected with WT MnSOD-Luc, ΔNF MnSOD-Luc, and ΔAP-1 MnSOD-Luc reporter plasmids, transfectants were either treated with LPS (+LPS) or left untreated (-LPS). Luciferase activity was normalized to untreated controls for WT MnSOD-Luc transfected cells.

MnSOD-Luc was not induced by LPS treatment. These data imply that the AP-1 binding site plays a role in LPS induction of MnSOD. In this set of experiments, ΔAP-1 MnSOD-Luc basal expression was not significantly different than that of WT MnSOD-Luc ($P=0.71$); however, in other experiments (Fig 18), ΔAP-1 MnSOD-Luc basal expression level was significantly reduced by 50% compared to WT MnSOD-Luc expression. Given this conflict, no conclusive statement can be made about the effect of the AP-1 deletion on basal expression. ΔNF MnSOD-Luc expression was not induced by LPS treatment ($P=0.45$) and the unstimulated level was reduced 3-fold compared to WT MnSOD-Luc expression ($P<0.001$). This finding implies that the NF-KB recognition site is important for maintaining basal transcription and for mediating the response to LPS.

4.10 Effect of A-Fos on Basal MnSOD Expression

To determine the requirement for AP-1 and NF-KB activities on basal MnSOD expression, the effect of A-Fos on MnSOD basal expression was tested by co-transfecting SVAREC cells with A-Fos and either WT MnSOD-Luc, ΔNF MnSOD-Luc, or ΔAP MnSOD-Luc (Fig. 18). A-Fos expression decreased WT MnSOD-Luc and ΔAP MnSOD-Luc expression by 42% and 60%, respectively (Fig. 18, compare columns 1 vs 2, and 3 vs 4). ΔNF MnSOD-Luc expression was not affected by A-Fos co-transfection. These data suggest that A-Fos inhibited basal expression of WT MnSOD and ΔAP-1 promoters. Since A-Fos decreases ΔAP MnSOD-Luc basal expression and the NF-KB site is present in the reporter, NF-KB may be necessary for basal levels of MnSOD expression.

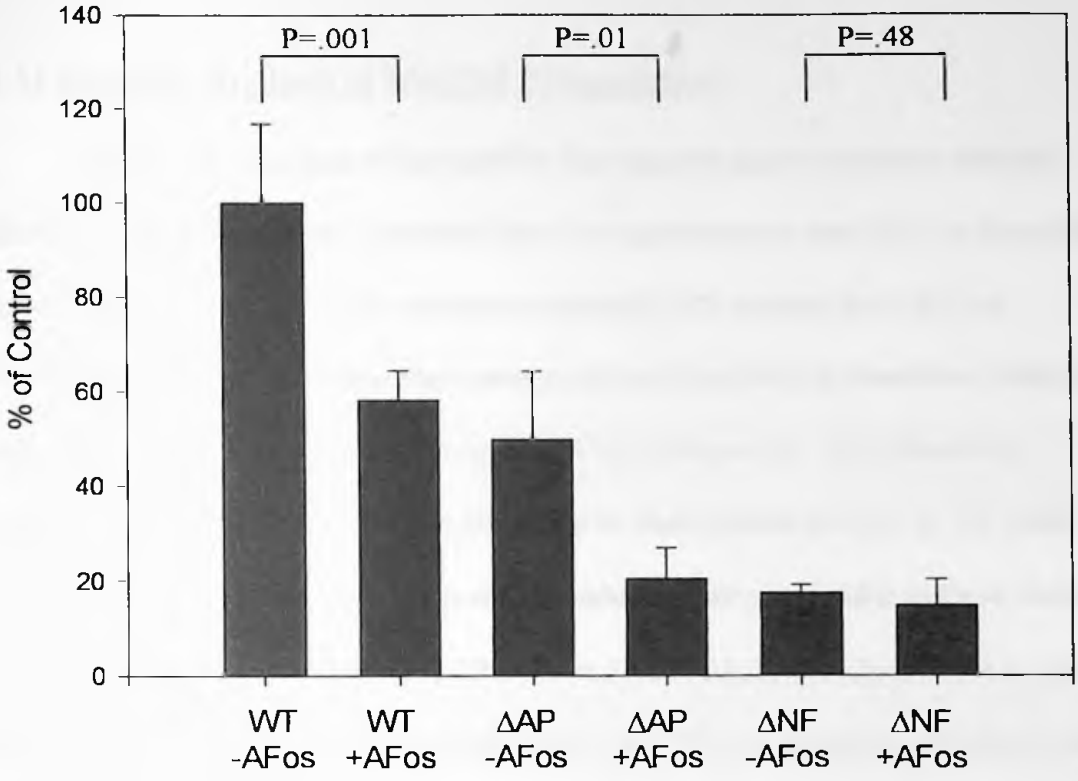


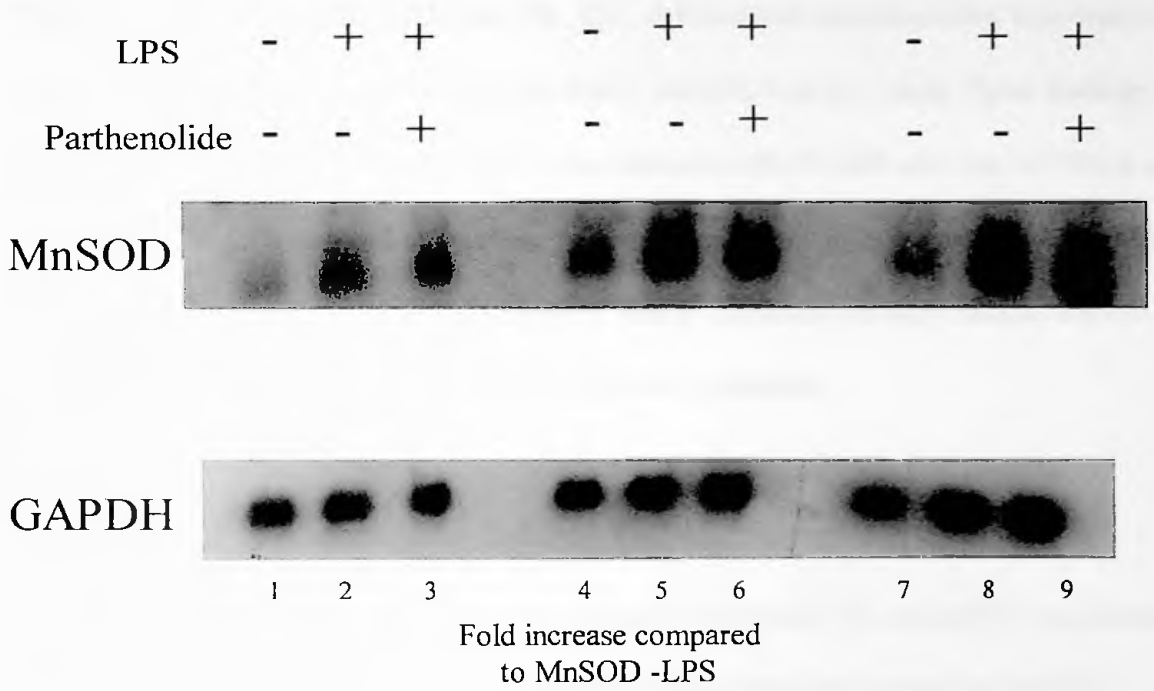
Figure 18.Effect of A-Fos on MnSOD reporter expression. SVAREC cells were transfected with WT MnSOD-Luc, ΔNF MnSOD-Luc, and ΔAP-1 MnSOD-Luc reporter plasmids and were co-transfected with either A-Fos (+A-Fos) or pGL-Basic (-A-Fos). Luciferase activity was normalized to untreated controls for WT MnSOD-Luc transfected cells.

4.11 Northern Analysis of MnSOD Transcription

In order to determine if the MnSOD-Luc reporter genes accurately reflected MnSOD mRNA abundance, a Northern blot was performed on total RNA collected from three different SVAREC cell populations: untreated, LPS treated, and LPS and parthenolide treated. Northern blots were probed with a cDNA derived from MnSOD exon 1 and mRNA abundance was quantitated by densitometry. LPS stimulation increased MnSOD mRNA 2.29-fold compared to unstimulated levels (Fig. 19, Lanes 1 vs 2, 4 vs 5, 7 vs 8). These increases in mRNA induction are comparable to those observed for WT MnSOD-Luc expression ($2.29 \pm .19$ vs $1.75 \pm .31$; $P = 0.1$ by Student's t test). Parthenolide decreased MnSOD stimulation by LPS 25% compared to LPS stimulated MnSOD (Fig. 19, Lanes 3 vs 2, 6 vs 5, 9 vs 8). In the presence of parthenolide mRNA levels were induced $1.73 \pm .25$ while reporter expression was induced $1.25 \pm .17$. Although these induction values are significantly different ($P=0.01$), the percent reduction measured by both methods (25% by Northern vs 28% by reporter assay) is very similar.

4.12 Effect of A-Fos on Induced WT MnSOD, ΔNF MnSOD and ΔAP MnSOD Reporter Expression

We observed that expression of the A-Fos protein could unexpectedly block expression of the NF-KB Luc reporter. One explanation for this finding is that A-Fos could dimerize with NF-KB to inactivate it, as it does with c-jun and c-fos. If so, then A-Fos protein should inhibit transcription of MnSOD reporters that had only a single



Fold increase compared
to MnSOD -LPS

1	2.29±.19	1.73±.25
MnSOD -LPS	MnSOD +LPS	MnSOD +LPS +Parth

Figure 19. Northern blot for detecting MnSOD. 10 μg of total RNA from SVAREC cells treated with LPS (Lanes 2, 4, and 6), LPS and parthenolide (Lanes 3,6, and 9) or left untreated (Lanes 1,4,and 7) were electrophoresed for 2 hours at 100 volts. The RNA was transferred to a positively charged nylon membrane and hybridized with a ^{32}P-labeled MnSOD DNA probe. The blot was then analyzed by autoradiography and densitometry.

recognition site, i.e. either an AP-1 or NF-KB site. To test this, SVAREC cells were co-transfected with the A-Fos expression plasmid and either WT MnSOD-Luc, ΔNF MnSOD-Luc or ΔAP-1 MnSOD-Luc (Fig. 20). A-Fos expression decreased expression of both WT MnSOD-Luc and ΔAP-1 (NF-KB+) MnSOD-Luc by 3-fold. These findings are consistent with the hypotheses that A-Fos interacts with NF-KB and that NF-KB is a requirement for LPS induced expression. A-Fos had no effect on expression of ΔNF-KB MnSOD-Luc expression (Fig. 20, compare 5^{th} and 6^{th} columns) perhaps because the NF-KB deletion profoundly reduced basal and induced expression.

4.13 Effect of Parthenolide on Induced MnSOD Reporter Expression

The effect of NF-KB on MnSOD regulation was indirectly assessed by expressing the A-Fos dominant negative protein. In order to more directly examine the NF-KB requirement, SVAREC cells were transfected with WT MnSOD-Luc, ΔAP-1 MnSOD-Luc, and ΔNF-KB MnSOD-Luc reporters and sequentially treated with parthenolide and LPS (Fig. 21). Parthenolide treatment decreased LPS stimulated WT MnSOD-Luc reporter expression by 40%. Parthenolide treatment had no significant effect on ΔNF MnSOD-Luc or ΔAP MnSOD-Luc expression. The reduction in WT MnSOD-Luc by parthenolide is consistent with an NF-KB requirement for MnSOD transcription, but a significant reduction was also expected in ΔAP MnSOD-Luc given that this reporter retains an NF-KB site.

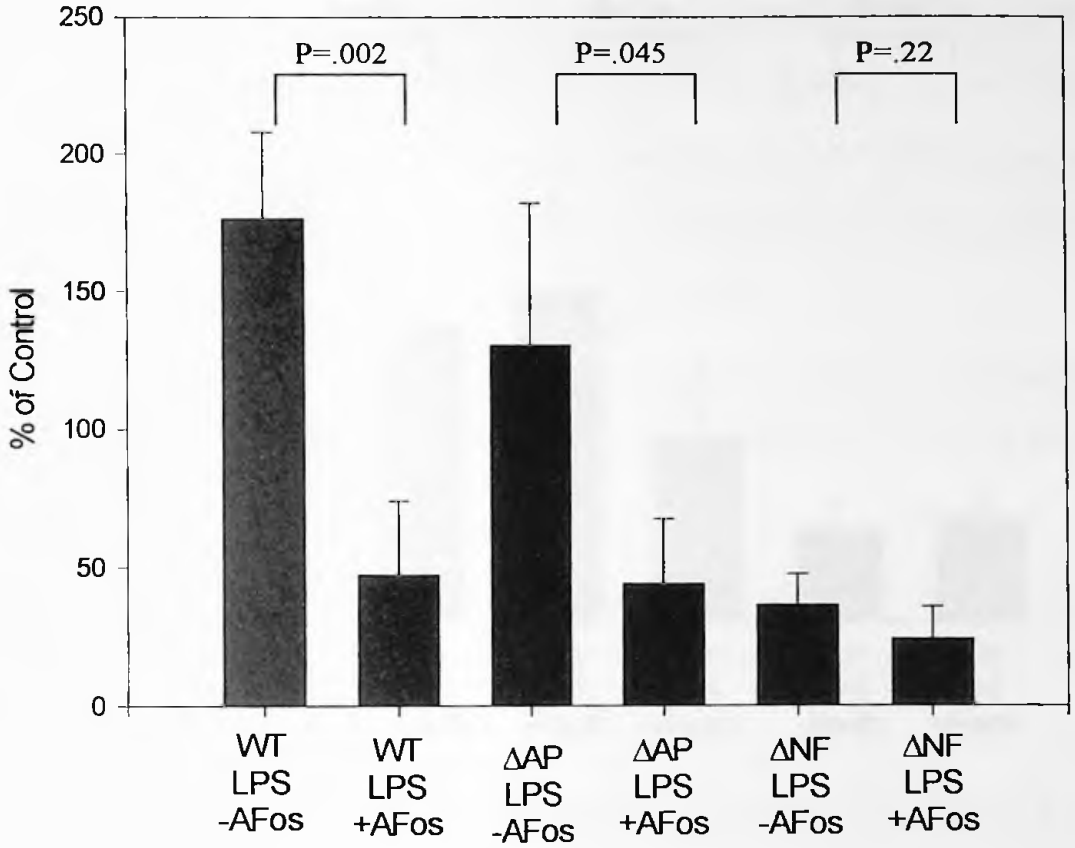


Figure 20. Effect of A-Fos on induced MnSOD reporter expression in SVAREC cells. SVAREC cells were transfected with WT MnSOD-Luc, ΔNF-MnSOD Luc, and ΔAP-1 MnSOD-Luc reporter plasmids, treated with LPS (+LPS) and co-transfected with either A-Fos (+AFos) or pGL-2 Basic (-AFos). Luciferase activity was normalized to untreated controls for WT MnSOD-Luc transfected cells.

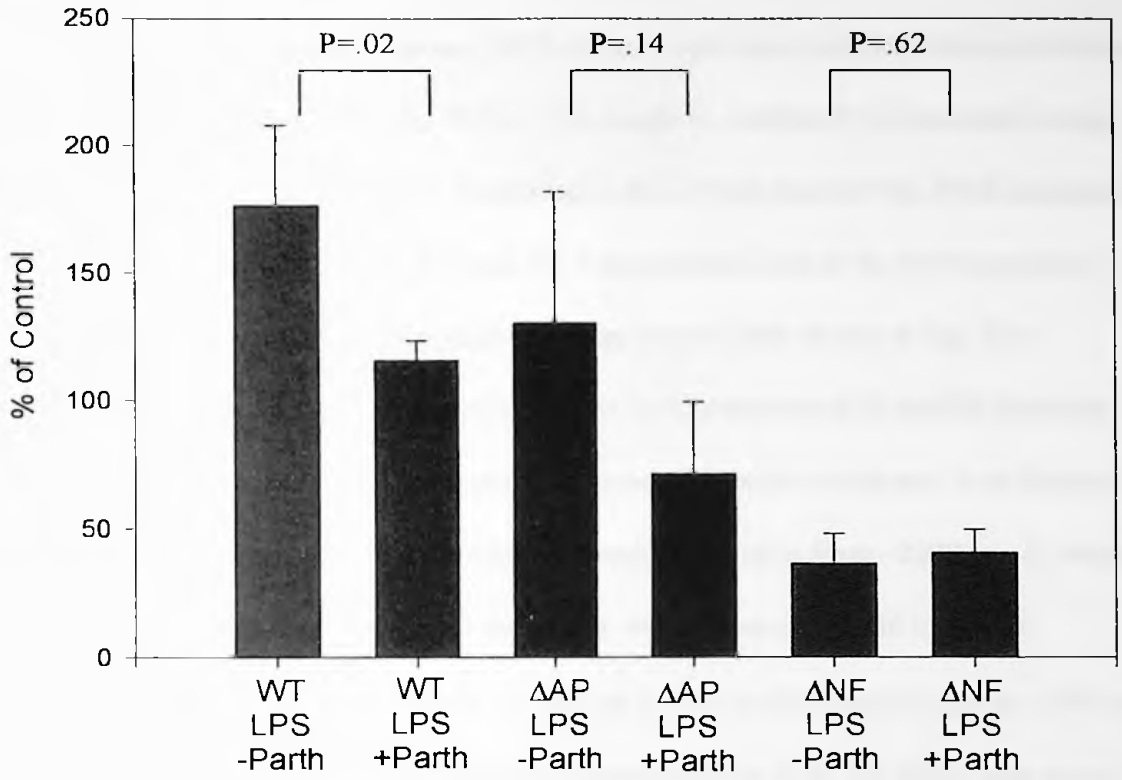


Figure 21. Effect of parthenolide on induced expression of MnSOD reporters in SVAREC cells. SVAREC cells were transfected with WT MnSOD-Luc, ΔNF MnSOD-Luc, and ΔAP-1 MnSOD-Luc reporter plasmids, stimulated with LPS (+LPS) and either treated with parthenolide (+Parth) or left untreated (-Parth). Luciferase activity was normalized to untreated controls for WT MnSOD-Luc transfected cells.

4.14 Identification of LPS response regions in iNOS promoters

LPS has been shown to induce iNOS in many cell types including the endothelium (Kifle et al., 1996; Moncada et al., 1991a). We sought to confirm this observation using INOS-Luc reporters and to identify transcription factors that mediate the iNOS response to LPS. The large number of NF-KB and AP-1 recognition sites in the iNOS promoter made deletion mutagenesis of individual sites impractical (sites shown in Fig. 22). Therefore, a series of iNOS-Luc nested deletions were constructed to identify essential regulatory regions in the iNOS promoter. The series of reporter constructs is as follows: (1) WT iNOS-Luc reporter, which contains promoter sequence from –3200 to –1, where –1 is the start of transcription, (2) 1.7 iNOS-Luc which contains –1746 to –1, (3) 0.7 iNOS-Luc which contains –680 to –1, and (4) 0.3 iNOS-Luc which contains –266 to –1 (Fig. 22). In order to determine if iNOS was stimulated by LPS, SVAREC cells were transfected with iNOS Luc reporters and treated with LPS. WT iNOS Luc expression was increased 2-fold by LPS stimulation, when compared to unstimulated levels (Fig. 23). This result suggests that iNOS transcription is induced by LPS treatment in SVAREC cells, and is consistent with previous studies which showed that LPS induced iNOS transcription in bovine endothelial cells (Xie et al., 1993). LPS treatment of SVAREC transfectants stimulated the 1.7 iNOS-Luc and 0.7 iNOS-Luc reporters 2-fold and 1.5-fold, respectively; the 0.3 iNOS-Luc showed no increase after LPS treatment (Fig. 23). Even though the 1.7 iNOS reporter was induced by LPS, the level of expression was decreased compared to basal WT iNOS. Since the 1.7 iNOS-Luc and 0.7 iNOS-Luc constructs retained inducibility, this suggests that the region from –3200 to –680

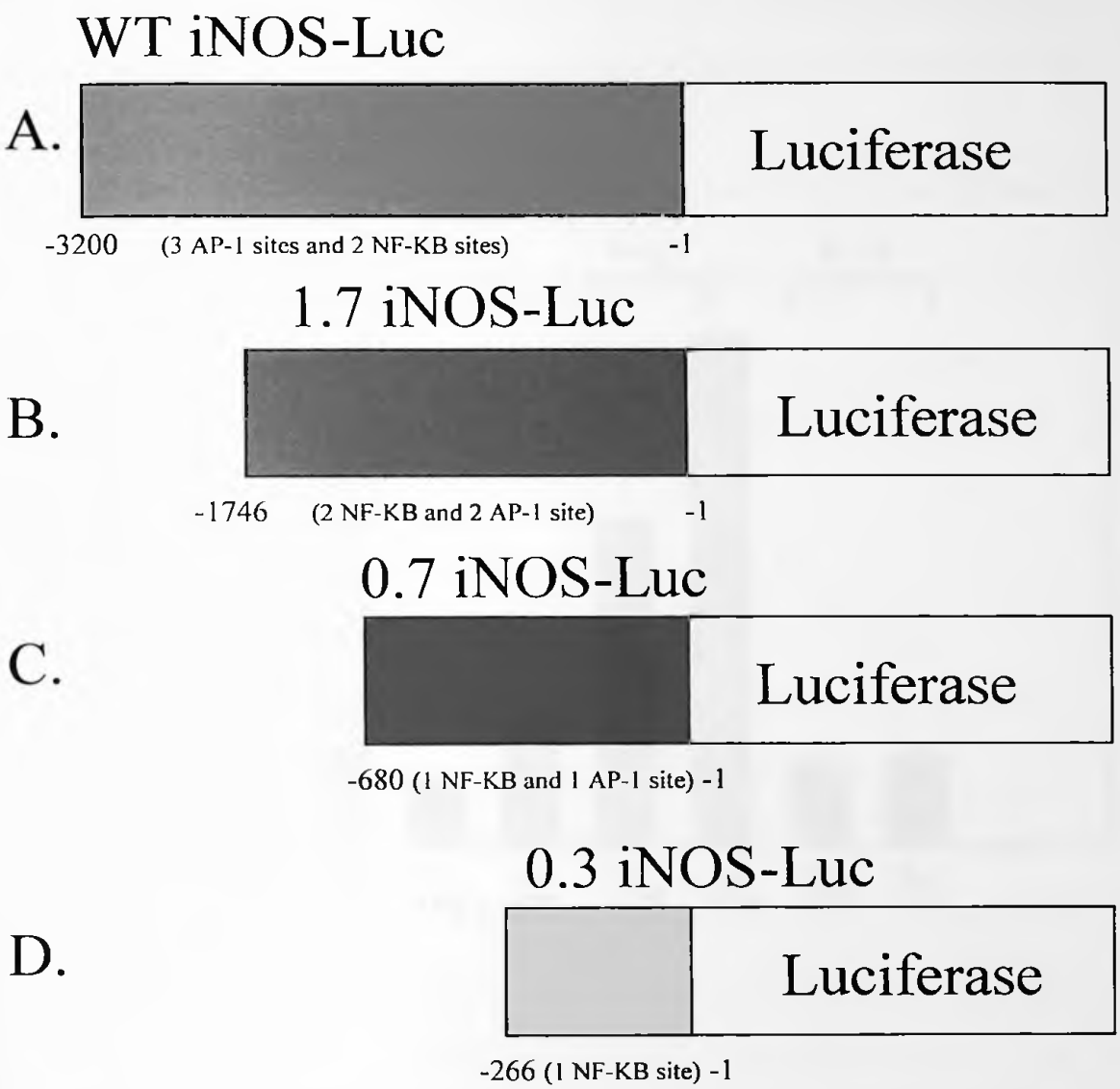


Figure 22. Schematic representation of the iNOS-luc reporter constructs. (A) WT iNOS-Luc contains 3 NF-KB and 2 AP-1 sites. (B) 1.7 iNOS-Luc contains 2 NF-KB and 2 AP-1 sites. (C) 0.7 iNOS Luc contains 1 NF-KB and 1 AP-1 sites. (D) 0.3 iNOS-Luc contains one NF-KB site. AP-1 sites are present at -2000 to –1993, -1127 to –1120, and -676 to –668; NF-KB sites are present at -956 to –966 and -104 to –111.

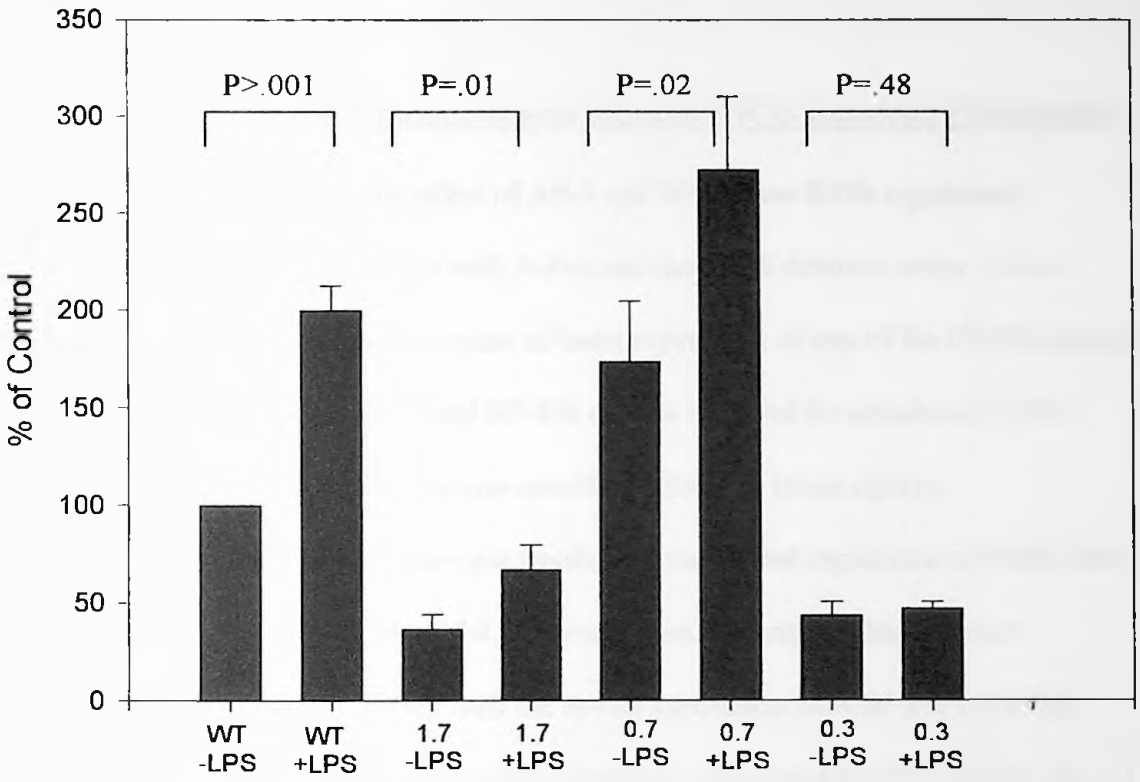


Figure 23. Effect of LPS on iNOS-Luc reporter expression. SVAREC cells were transfected with WT iNOS-Luc, 1.7 iNOS-Luc, 0.7 iNOS-Luc and 0.3 iNOS-Luc reporter plasmids and either treated with LPS (+LPS) or left untreated (-LPS). Luciferase activity was normalized to untreated controls for WT INOS-Luc transfected cells.

does not play a large role in iNOS regulation and that there is a sequence between –680 and –266 required for LPS induction. Promoters below 680bp cannot maintain induction of transcription, as shown by the 0.3 iNOS-Luc reporter.

4.15 Effect of A-Fos on Uninduced and Induced iNOS Reporter Expression

In order to determine the effect of AP-1 and NF-KB on iNOS regulation, SVAREC cells were co-transfected with A-Fos and the iNOS deletion series. A-Fos expression caused no significant decrease of basal expression of any of the iNOS deletions (Fig. 24). This suggests that AP-1 and NF-KB are not required for uninduced iNOS transcription and that A-Fos is not a non-specific inhibitor of transcription.

Since AP-1 and NF-KB were not involved in uninduced regulation of iNOS, were they necessary for induced levels of iNOS transcription. To answer this question, SVAREC cells were co-transfected with the A-Fos expression plasmid and the iNOS deletion series and treated with LPS. A-Fos expression decreased the WT iNOS-Luc, 1.7 iNOS-Luc and 0.7 iNOS-Luc reporter expression, but had no effect on the 0.3 iNOS-Luc reporter expression (Fig. 25). These data indicate that AP-1 and NF-KB are important to iNOS expression, but do not determine the importance of AP-1 or NF-KB individually. The largest decrease produced by A-Fos on LPS treatment was with the 0.7 iNOS-Luc, which further supports the previous findings that there is a critical sequence between –680 and –266 that is necessary for iNOS transcriptional induction.

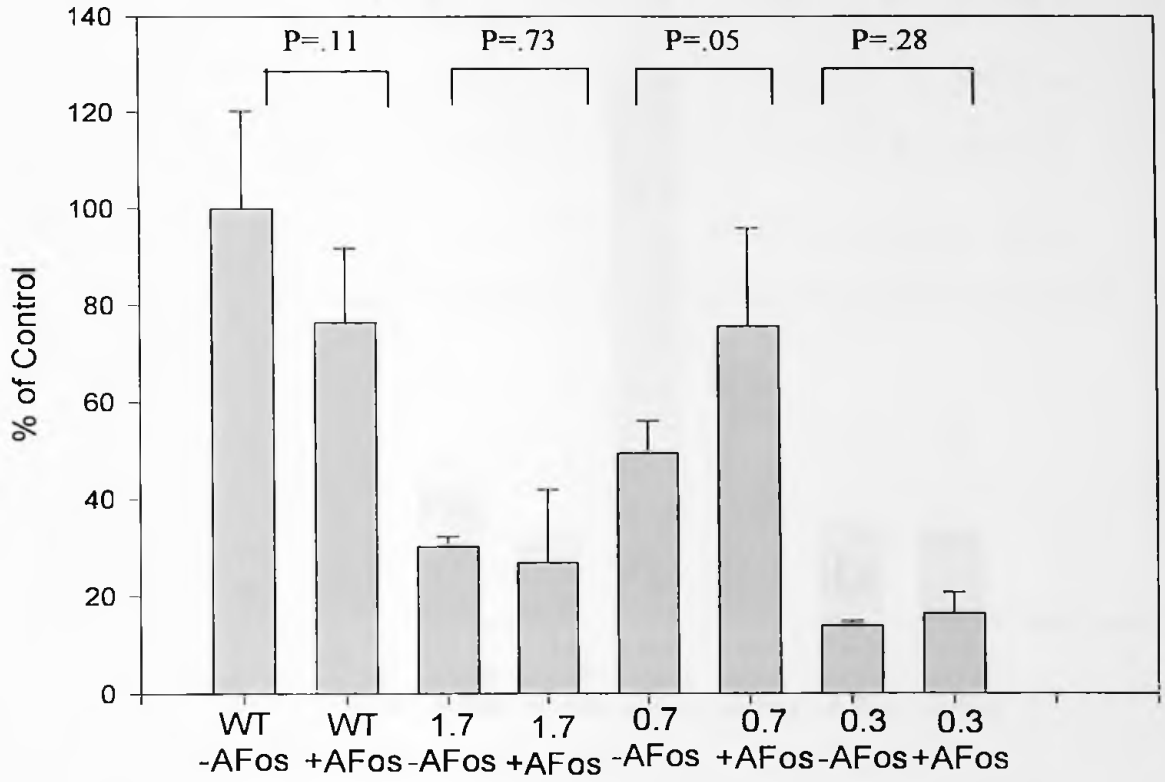


Figure 24. Effect of A-Fos expression on uninduced iNOS-Luc expression. SVAREC cells were transfected with WT iNOS-Luc, 1.7 iNOS-Luc, 0.7 iNOS-Luc and 0.3 iNOS-Luc reporter plasmids and either co-transfected with A-Fos plasmid (+A-Fos) or pGl-Basic (-A-Fos). Luciferase activity was normalized to untreated controls for WT iNOS-Luc transfected cells.

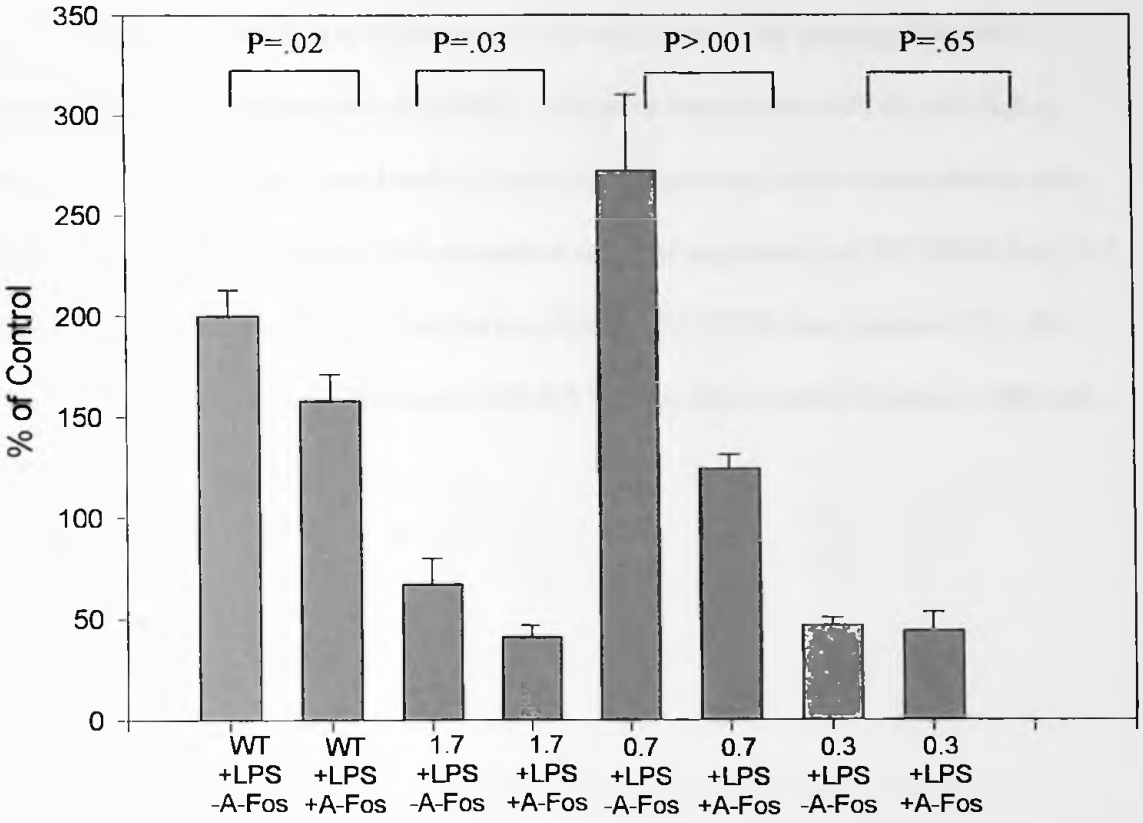


Figure 25. Effect of A-Fos expression on induced iNOS-Luc reporter expression. SVAREC cells were transfected with WT iNOS-Luc, 1.7 iNOS-Luc, 0.7 iNOS-Luc and 0.3 iNOS-Luc reporter plasmids and either co-transfected with A-Fos (+A-Fos) or pGL-Basic (-A-Fos) and stimulated with LPS (+LPS). Luciferase assays were normalized to untreated controls for WT INOS Luc transfected cells.

4.16 Effect of Parthenolide on Induced iNOS Reporter Expression

The role of NF-KB in iNOS regulation was assessed by treating SVAREC transfectants with parthenolide. SVAREC cells were transfected with the iNOS-Luc deletion series and then treated with parthenolide 30 minutes prior to stimulation with LPS. Parthenolide decreased LPS stimulated reporter expression of WT iNOS-Luc, 1.7 iNOS-Luc, and 0.7 iNOS-Luc, but had no effect on 0.3 iNOS-Luc reporter (Fig. 26). This suggests that there are functional NF-KB binding sites located between –680 and –266 in the iNOS promoter.

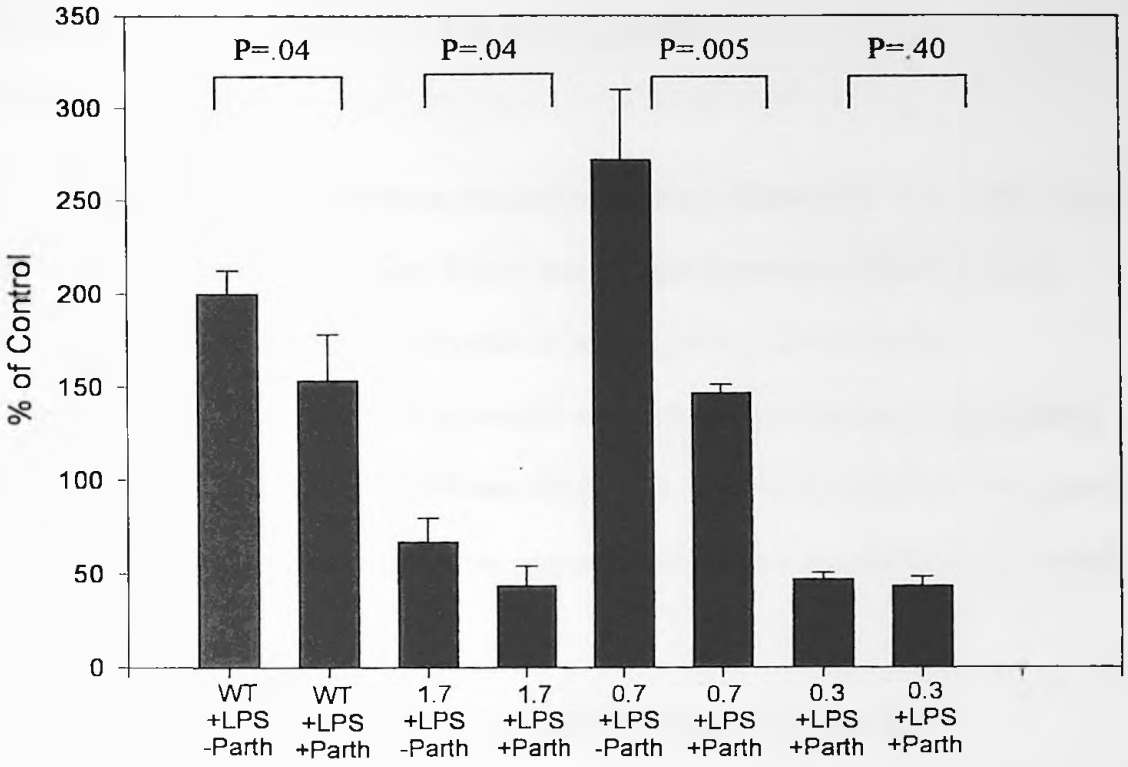


Figure 26. Effect of parthenolide on induced iNOS Luc reporter expression. SVAREC cells were transfected with WT iNOS-Luc, 1.7 iNOS-Luc, 0.7 iNOS-Luc and 0.3 iNOS-Luc reporter plasmids, stimulated with LPS (+LPS) and pretreated with either Parthenolide (+Parth) or untreated (-Parth). Luciferase assays were normalized to untreated controls for WT iNOS- Luc transfected cells.

5.0 Discussion

Since MnSOD and iNOS are induced by common stimuli (Xie et al., 1993; Kuo et al., 1999), we sought to determine if there was a single transcription factor or set of factors which was necessary for induction of both genes in endothelial cells. A comparison of MnSOD and iNOS promoters revealed that both promoters had binding sites for transcription factors NF-KB and AP-1. This study was performed to determine if either NF-KB or AP-1 are necessary for transcriptional regulation of MnSOD and iNOS.

5.1 Regulation of NF-KB, AP-1, and MnSOD in PRAEC cells

The first task was to prepare primary endothelial cell cultures from rat aortae. Because the endothelial cell produces NO which causes smooth muscle relaxation, endothelial cells are the appropriate target for study of iNOS and MnSOD regulation. The original protocol published by McGuire and Orkin (1987) did not produce pure cultures in my hands, as they contained smooth muscle cells as determined by western blotting (Fig. 4). An optimized protocol, which gave rise to pure endothelial cell cultures was used to create PRAEC cells, which were used to assess the roles of NF-KB and AP-1 in MnSOD regulation.

To determine if NF-KB and AP-1 were regulated in PRAEC cells, luciferase reporter vectors AP-1 Luc and NF-KB Luc were transfected into PRAEC cells, which were either treated with LPS or left untreated. Reporter expression reflects transcriptional

activation of AP-1 or NF-KB and the activity of these activators. Therefore, the level of luciferase expression measures the amount of transcription factor activity. Since LPS treatment increased AP-1 and NF-KB reporter expression, LPS induced activity of both transcription factors in PRAEC cells. LPS treatment of PRAEC cells induced the WT MnSOD-Luc reporter, which showed that LPS treatment induces expression of MnSOD. This finding shows that MnSOD transcription can be induced in PRAEC cells, which is similar to other studies in porcine endothelial cells (Visner et al., 1991).

PRAEC cultures were not optimal for experimentation because their growth rate was very slow. A six-week growth period was required to generate sufficient cells for a single experiment. In order to facilitate completion of experiments in a timely manner, an alternative endothelial cell line was sought.

5.2 SVAREC Cell Characterization

SVAREC cells grew at an appropriate rate (doubling once every day), and cells were split every 4-5 days. In order to ascertain their endothelial nature, SVAREC cells were tested for the presence of vWF and α-actin by western blotting. vWF and α-actin were both present. The presence of vWF confirms that SVAREC are derived from an endothelial lineage. Detection of α-actin by western blot could have been caused by smooth muscle contamination of the SVAREC cultures or by recognition of an actin isoform. In confocal microscopy studies, α-actin antibody was bound to targets within the outer cell membrane of SVAREC cells (Fig.7, A and B) but not the typical α-actin fibers

found in smooth muscle cells like the A7r5 line (Fig.7 D). Most likely the antibody is interacting with gamma-actin, or some other actin isoform in these endothelial cells. This finding supported the vWF western blot evidence that SVAREC cells were endothelial and the confocal evidence showed that they were not contaminated with smooth muscle.

5.3 NF-KB and AP-1 Regulation in SVAREC Cells

LPS regulation of NF-KB and AP-1 activity was tested by transfecting SVAREC cells with AP- Luc and NF-KB Luc reporter vectors. LPS treatment stimulated expression of both the AP-1 and NF-KB reporter vectors (Fig. 13 and 14), showing that LPS can activate both transcription factors in SVAREC cells.

To determine if the response of the reporter vectors was specific for NF-KB and AP-1 expression, SVAREC transfectants were treated with inhibitors of NF-KB or AP-1. AP-1 activity was increased by LPS treatment, not affected by parthenolide and inhibited by the expression of A-Fos. These results were to be expected, since it was known that A-Fos decreases AP-1 activity (Olive et al., 1997), and parthenolide should not affect AP-1 activity. A second finding, also anticipated, was that treatment of SVAREC transfectants with parthenolide before exposure to LPS decreased NF-KB Luc expression (Fig. 14). This showed that the NF-KB reporter was responding to NF-KB.

A-Fos expression unexpectedly resulted in a near complete inhibition of the NF-KB Luc expression (Fig. 14). A-Fos has been shown to have no effect on other transcription factors such as CREB and GRE (Olive et al., 1997). There are two possible explanations for these observations. The first explanation is that AP-1 activation is

required for NF-KB activation. Thus, when AP-1 is inhibited by A-Fos, NF-KB would remain inactive. A second possible mechanism is based on the finding that proteins from the Rel and b-Zip families can interact (Stein et al., 1993a). NF-KB which has a rel family subunit, can bind to C/EBP, a b-Zip protein. It is possible that NF-KB can interact with other b-Zip proteins like AP-1. Since the A-Fos protein retains the b-Zip motif, it could bind to the rel domain of NF-KB and physically prevent NF-KB from activating transcription.

5.4 MnSOD Regulation in SVAREC Cells

LPS treatment of SVAREC cells induced the WT MnSOD-Luc reporter 1.75 ± 0.31 fold (Fig. 15), and MnSOD mRNA was increased 2.29 ± 0.19 fold (Fig. 16). Since there was no significant difference (P=.10) between the two methods as compared by the Student's t test, these results validate the use of the luciferase reporter constructs as a measure of mRNA abundance. The observed induction of MnSOD in SVAREC cells supports the use of these cells as an endothelial model because they responded to LPS treatment in the same manner as the PRAEC cells.

The MnSOD promoter region contained only one copy of the AP-1 and NF-KB binding sites. The specific effect of each transcription factor was tested by deleting only the DNA recognition site for NF-KB and AP-1 in the MnSOD promoter region. Introduction of the NF-KB deletion into WT MnSOD-Luc reduced basal and induced expression of the reporter gene (Fig. 17). This observation indicates that MnSOD transcription is reliant upon NF-KB for basal and induced regulation. This finding

correlates with another study using lung fibroblasts stimulated by IL-1, which found that NF-KB was the critical transcription factor necessary to maintain transcriptional induction by TNF-α and IL-1 (Xu et al., 1999). It cannot be determined if MnSOD basal regulation requires AP-1, because there were two conflicting results discovered in this study (Fig. 17 column 3 vs Fig. 18 column 3). However, when the ΔAP-1 MnSOD-Luc reporter was stimulated by LPS, there was no increase in reporter activity compared to unstimulated ΔAP-1 MnSOD-Luc (Fig. 17). This implies that both AP-1 and NF-KB are needed for MnSOD induction by LPS.

The levels of reporter expression in ΔNF-KB MnSOD-Luc are 90% below uninduced WT MnSOD and are not stimulated by LPS (Fig. 18). Thus, the deletion of the NF-KB binding site from the MnSOD promoter region prevents uninduced and induced transcription, and indicates that this site is critical for the regulation of MnSOD. In addition, A-Fos expression produced no effect on basal or LPS treated ΔNF-KB MnSOD-Luc (Fig. 18 and 19), further supporting NF-KB as the critical transcription factor necessary for basal and induced MnSOD transcription.

A-Fos expression appears to inhibit both AP-1 and NF-KB. A-Fos expression decreased basal expression of WT MnSOD-Luc and ΔAP MnSOD-Luc, which suggests that basal regulation of MnSOD is reliant upon NF-KB and/or AP-1. Since the ΔAP-1 MnSOD-Luc reporter retains one NF-KB element, the decrease in reporter expression by A-Fos could be due to the inhibition of NF-KB. The observation that A-Fos expression decreases the LPS stimulated expression of the ΔAP MnSOD-Luc reporter suggests that A-Fos expression inhibited a transcription factor necessary for induction of MnSOD (Fig.

21). Since NF-KB is present in the ΔAP MnSOD-Luc reporter, the inhibition of NF-KB could be that necessary transcription factor needed for MnSOD induction by LPS. Therefore, NF-KB is required for basal transcription of MnSOD and induction of MnSOD by LPS requires both NF-KB and AP-1 transcription factors.

In order to support the finding that MnSOD induction requires NF-KB, MnSOD regulation was tested by treating SVAREC transfectants with parthenolide. Parthenolide treatment decreased LPS stimulation of WT MnSOD-Luc by 40% and ΔAP MnSOD-Luc by 45% but had no effect on ΔNF-KB MnSOD-Luc expression (Fig. 21). The decrease in WT MnSOD-Luc and ΔAP MnSOD-Luc by parthenolide is consistent with the presence of a functional NF-KB site in these promoters. There is a noticeable difference between the effect of A-Fos on MnSOD expression and inhibition of NF-KB by parthenolide. Parthenolide only partially inhibits NF-KB, thus allowing some active NF-KB to bind and facilitate transcription, while A-Fos is completely inhibiting NF-KB activity. This is shown by parthenolide inhibiting LPS stimulated MnSOD reporter expression by 40%, compared to 90% inhibition with A-Fos expression. It has been shown that parthenolide does not completely inhibit NF-KB (Hehner et al., 1998); thus, some active NF-KB remains in the cell which could account for this difference in inhibition of MnSOD.

The finding that MnSOD regulation is reliant upon NF-KB agrees with the work of Xu et al. (1999), which states that NF-KB is the transcription factor required for MnSOD induction by TNF-α and IL-1. However, it does contradict another study, which stated that NF-KB was not required for transcription of MnSOD (Bedoya et al., 1995). This study treated RINmF5 cells with IL-1 alone or in combination with PDTC. It was found

that PDTC treatment did not reduce the levels of MnSOD mRNA. Thus, the authors conclude that NF-KB is not critical for IL-1 mediated MnSOD induction. The apparent contradiction between these studies could be due to different regulation of MnSOD in different cell types, or the effect of different stimulators on MnSOD regulation.

MnSOD contains no TATA or CAAT boxes (Wan et al., 1994, Kuo et al., 1999), which are generally accepted as the initiators of transcription. Therefore, MnSOD must contain another mechanism that allows the basal transcriptional components to form and initiate transcription. Because the NF-KB deletion decreased basal regulation by 90% and prevented any induction from that level by LPS treatment, I speculate that NF-KB involvement with MnSOD could play a role in recruiting the basal apparatus to the promoter.

5.5 iNOS Regulation in SVAREC Cells

iNOS is an extremely important enzyme that helps maintain vascular tone. It has been studied thoroughly in several cell types and several studies reflect that iNOS regulation is dependent upon the transcription factor NF-KB (Spink et al., 1995; Zhang et al., 1998; Xie et al., 1993). We were interested in studying the regulation of iNOS in endothelial cells in coordination with MnSOD. The WT iNOS contains 2 NF-KB sites (-956 to -966; -104 to –111) and 3 AP-1 sites (-2000 to -1993; -1127 to -1120; and -676 to -668) (Fig. 22A). Because there were too many AP-1 and NF-KB sites to specifically delete, a series of nested promoter deletions (1.7 iNOS, 0.7 iNOS, and 0.3 iNOS) were generated in order to locate sites required for LPS induction. Since the 0.7 iNOS reporter

produced increased basal and inducible levels by LPS treatment, while the 0.3 iNOS reporter produced reduced basal and no induction upon LPS treatment (Fig. 23). There are NF-KB response elements between –680 and –266 that are important for iNOS transcriptional induction, or there is a DNA site necessary for NF-KB induction by LPS.

A second important aspect of iNOS regulation is shown by the 1.7 iNOS-Luc construct compared to the WT iNOS-Luc and 0.7 iNOS-Luc. Expression of the 1.7 iNOS-Luc reporter expression was diminished compared to the WT iNOS-Luc and 0.7 iNOS-Luc (Fig. 23). A possible explanation for this decrease could be that an inhibitory DNA element is active in 1.7 iNOS-Luc, but not functional in the WT iNOS-Luc. The deletion from –1746 to –680 removes this inhibition so expression of 0.7iNOS-Luc is higher than the WT iNOS-Luc or 1.7 iNOS-Luc. Data from this study implies that a negative regulatory element for iNOS transcription is present between –1746 and –680 of the iNOS promoter.

When A-Fos was expressed in SVAREC transfectants, no decrease in basal iNOS-Luc levels was observed (Fig. 24). This finding suggests that NF-KB and AP-1 are not necessary for basal levels of transcription of iNOS. Furthermore, this shows that A-Fos was not a general transcriptional inhibitor. However, when SVAREC cells were co-transfected with the iNOS-Luc series and A-Fos, then treated with LPS, a marked decrease in stimulation of WT iNOS-Luc, 1.7 iNOS-Luc, and 0.7 iNOS-Luc reporters was found (Fig. 25). These data imply that either or both NF-KB and AP-1 are important for iNOS induction, but not basal regulation.

Parthenolide treatment of iNOS Luc transfected cells produced a marked decrease in the WT iNOS-Luc, 1.7 iNOS-Luc, and 0.7 iNOS-Luc reporter constructs (Fig. 26). The largest decrease was found in the 0.7 iNOS reporter, where parthenolide treatment decreased the level of expression 42%. There is a NF-KB consensus site present at –114 in the iNOS promoter, thus this binding site could be necessary for iNOS induction by LPS. These data suggest that NF-KB has a role in iNOS transcriptional induction, and that the most important NF-KB site for induction is within 680 bp from the transcription start site.

Overall, the analysis of iNOS has suggested that NF-KB is an important transcription factor for iNOS induction. This follows previous studies that suggest the same result in other cells types (macrophages and smooth muscle) (Lowenstein et al., 1993; Spink et al., 1995; Zhang et al., 1998). Therefore, the findings here are not unexpected and re-enforce the previous studies. It also suggests that iNOS regulation is dependent upon NF-KB regardless of the species or cell type, in other words NF-KB is a universal requirement for iNOS transcriptional induction.

This study was interested in looking at MnSOD regulation and iNOS regulation in endothelial cell, specifically by the transcription factors NF-KB and AP-1. It was found that both NF-KB and AP-1 were necessary for MnSOD induction by LPS; however, there was not conclusive evidence that AP-1 did or did not have an effect on iNOS. Therefore it can only be concluded that AP-1 is necessary for induction of MnSOD by LPS, and it could play a role in iNOS regulation.

In conclusion, findings from this study imply NF-KB as critical transcription factor needed to maintain both MnSOD and iNOS regulation. NF-KB is needed for the basal regulation of MnSOD and is necessary for both iNOS and MnSOD induction. Therefore, the balance between O_2^- and NO could be affected by the transcription factor NF-KB. When NF-KB is active it facilitates the transcription of iNOS and MnSOD, which will allow dismutation of O_2^- and allow NO to diffuse into the smooth muscle and direct vasodilation. If NF-KB is not active, MnSOD is not transcribed and O_2^- will be available to react with NO and form $OONO^-$, which will prevent activation of GC and facilitate vasoconstriction.

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