Oxidative stress activates the plasminogen activator inhibitor type 1 (PAI-1) promoter through an AP-1 response element and cooperates with insulin for additive effects on PAI-1 transcription.

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ABSTRACT

Oxidative stress is one of the characteristics of diabetes and is thought to be responsible for many of the pathophysiological changes caused by the disease. We previously identified an insulin response element in the promoter of plasminogen activator inhibitor 1 that was activated by an unidentified member of the forkhead/winged helix (Fox) family of transcription factors. This element mediated a 5- to 7-fold increase in PAI-1 transcription due to insulin. Here we report that oxidative stress also caused a 3-fold increase in PAI-1 transcription and that the effect was additive with that of insulin. Antioxidants prevent this response. Mutational analysis of the PAI-1 promoter revealed that oxidative stress acted at an AP-1 site at -60/52 of the promoter. Gel-mobility shift analysis demonstrated that binding to an AP-1 oligonucleotide was increased 4-fold by oxidative stress. Jun levels were increased by oxidants as assessed by RT-PCR. Western blotting demonstrated that a rapid and prolonged nuclear accumulation of phospho-c-Jun followed oxidant stimulation. The nuclear c-Jun phosphopylation was not observed in cells treated with reduced glutathione. Finally, JNK/SAPK activity was found to increase in response to oxidants and inhibition of JNK/SAP blocked TBHQ-increased PAI-1-Luciferase expression. Thus, oxidative stress stimulated AP-1 and activated the PAI-1 promoter.
INTRODUCTION

PAI-1 levels in the blood are elevated in diabetes due to increased PAI-1 production (1-4). The elevated PAI-1 levels may contribute to the macro and micro vascular complications of the disease (5). Numerous factors were shown to activate production of PAI-1 including insulin (6), PDGF (7), FGF (7), IL-1α, TGF-β (8), angiotensin II (9), TNF-α (10), thrombin (11), and oxidation products (12) while IFN-γ (13) inhibited PAI-1 production. Many of these could be altered in diabetes, but increased levels of insulin and oxidative stress are of primary importance.

We previously demonstrated that Insulin activated the PAI-1 promoter through a forkhead-related response element (TTATTT) at -52/-43 of the PAI-1 promoter (14). Insulin-increased PAI-1 gene expression was inhibited by the expression of the DNA binding domain of forkhead. Finally, LexA-FKHR increased the expression of a LexA-CAT reporter in insulin treated cells. Thus, it appeared that a FKHR related transcription factor mediated the effect of insulin on the PAI-1 gene. The FKHR-related factor was probably not FKHR (FoxO1) itself since FKHR was expressed in both insulin-responsive and non-responsive cell types.

Oxidative stress is a characteristic of diabetes. This is partially the result of increased blood glucose. The major cause of protein oxidation in diabetes is keto aldehydes and oxidizing intermediates that are formed under in vivo conditions by a reaction between glucose and oxygen (15). Additionally, excess
glucose is shunted through the aldose reductase pathway. This depletes NADPH reducing potential and leaves antioxidants in their oxidized state (16).

How cells sense oxidative stress arising from a multitude of different compounds was not completely determined. Oxidants activate the transcription of genes for antioxidant defense primarily through stimulation of either NF-kB or AP-1 (17-20).

The additive activation of PAI-1 gene transcription by Insulin and oxidative stress could explain much of the increase in circulating PAI-1 in diabetics. These experiments sought to define that response. An AP-1 element in the PAI-1 proximal promoter mediated activation of PAI-1 gene transcription by oxidants.

Gel-mobility shift experiments demonstrated an oxidation responsive increase in a factor that binds the AP-1 element. RT-PCR showed that c-Jun is increased by oxidative stress. Western blot analysis demonstrated that oxidative stress increased the phosphorylation of JNK/SAP and nuclear localized c-Jun.

MATERIALS AND METHODS

Materials - [\(^{32}\)P]dGTP, 3000 Ci/mmol, was obtained from ICN Biochemicals Corporation. Oligonucleotides were from Operon and reagents for PCR were obtained from Roche. Medium components were obtained from Hyclone Laboratories. Antibodies to c-Jun, phospho-c-Jun, JNK/SAP and phospho-JNK/SAP were from Cell Signaling Technologies while horseradish peroxidase conjugated goat anti rabbit secondary antibody was from Upstate. Inhibitors were from Calbiochem. All other reagents were of the highest purity.
available and were obtained from Sigma, Calbiochem, Bio-Rad, Eastman, Fisher, or Roche.

Plasmids - The PAI-1 promoter reporter plasmid, p800neo-Luc, was the generous gift of Dr. D. Rifkin (NYU School of Medicine) (21). Chloramphenicol acetyltransferase (CAT) reporter plasmids were constructed from p800neo-Luc by polymerase chain reaction (PCR) as previously described (22). Deletion mutants of this plasmid were made by PCR and point mutations of these plasmids were also made by PCR using mutant primers as described (14,23). The human insulin expression vector, pRT3HIR2, was the gift of Dr. J. Whittaker (Hagedorn Institute, Copenhagen, Denmark).

Transient Gene Transfection Facilitated by Electroporation -
Electroporation experiments and reporter assays were performed as described (24). GH4 cells were harvested with an EDTA solution, and 20 to 40 x 10^6 cells were used for each electroporation. All electroporations contained 5 µg of the plasmid pHIR-RT3 that expresses high levels of the human insulin receptor (25). This is necessary to achieve the high levels of insulin stimulation seen in these studies and is consistent with numerous other systems where co-transfection of receptors has been necessary to achieve physiological regulation of transfected genes (25). Experiments with GH4 cells stably transfected with the human insulin receptor give similar results. Trypan blue exclusion before electroporation ranged from 95% to 99%. The voltage of the electroporation was 1550 volts. This gives trypan blue exclusion of 70% to 80% after electroporation. The
transformed cells were then plated in multiwell dishes (Falcon Plastics) at \(5 \times 10^6\) cells per 9-cm\(^2\) tissue culture well in DMEM with 10% hormone-depleted serum (26,27). Cells were refed at 24 h with DMEM with 10% hormone depleted serum \(\pm\) insulin (1\(\mu\)g/ml bovine insulin, Calbiochem). After 48 h, the flasks were washed three times with normal saline and frozen. The cells were harvested and reporter activity was assayed. Luciferase assays were performed on GH4 cell lysates using reagents and protocols from Promega. Luciferase activity was normalized for variability of transfections using \(\beta\)-galactosidase as described below. Control experiments demonstrated that stimulation of PAI-1-Luciferase by insulin and other hormones was identical to that seen with the corresponding CAT reporter (data not shown).

An RSV-\(\beta\)-galactosidase expression plasmid was included in the electroporations. This plasmid is not expressed and its inclusion has no effect on the overall results of the experiments, but it was included to control for minor variations in transfection efficiency. Briefly, 2 \(\mu\)g of RSV-\(\beta\)-galactosidase expression plasmid was included in the electroporations. The \(\beta\)-galactosidase activity in the cell lysates was determined using \(o\)-nitrophenyl-\(\beta\)-D-galactopyranoside. Transfection efficiency did not vary significantly among transfections performed at the same time. The % acetylation was then corrected for minor variations in \(\beta\)-galactosidase activity by converting the % acetylation to % acetylation /OD430 \(\beta\)-galactosidase activity/mg protein. The fold stimulation or
inhibition was then determined. Statistical analysis was performed on all experiments using Student's t-test.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) of Fos and Jun – mRNA was prepared using a filter binding protocol (Trevigen). The amount of mRNA was estimated from the absorbance at 260/280 nm. The ratio of the optical density at 260 nm to the optical density at 280 nm was generally 2 or greater. Approximately equal amounts of mRNA (~0.1 µg) were used with primers for the mRNA of glyceraldehyde-3-phosphate dehydrogenase in a single tube RT-PCR assay (Tetra Link, Amherst, NY). The RT-PCR product was quantitated using a Fluoroimager 575 and ImageQuant software (Amersham-Pharmacia Biotech). Equal amounts of mRNA (based on GAPDH signal) were then used for assay of FKHR mRNA. Primers for RT-PCR of c-Fos were 5'-CGTTGCAGACTGAGATTGCC-3' (sense) and 5'-ACCGGACAGGTCCACATCTG-3' (antisense). Primers for RT-PCR of c-Jun were 5'-AACTCGGACCTTCTCACGTCG-3' (sense) and 5'-TGCTGAGGTTGGCGTAGACC-3' (antisense). Primers for RT-PCR of rat PAI-1 were 5'-GCCTCCAAAGACCGAAATGTG-3' (sense) and 5'-GTCGTTGATGATGAATCTGGCTC-3' (antisense). The products were sequenced to verify that they were c-Fos, c-Jun, or PAI-1.

Assay of DNA-Protein Binding by Gel Electrophoresis - An oligonucleotide containing an AP-1 response element was prepared, annealed, purified on polyacrylamide-gels, and end labeled with $[^{32}\text{P}]$dGTP using the Klenow fill-in
reaction. The sequence of this oligonucleotide is 5'-GATCCTGACTCAGCGC -3'. 
A mutant oligonucleotide with the sequence 5'- GATCCAGaCACaGCGC -3' was 
also prepared. A similar mutation in the AP-1 response element of the PAI-1 
promoter abolishes oxidant responsiveness of the PAI-1 promoter. Labeled AP-1 
response element DNA was then used in mobility-shift experiments with 
unlabeled nuclear extracts performed as described (24). Three µg of nuclear 
extract was incubated at 25°C for 30 min. with 30,000 cpm (10 to 20 fM) of 32P-
labeled PAI. The protein-DNA complexes were then analyzed by electrophoresis 
on a 6% polyacrylamide gel in 25 mM Trisma, 25 mM boric acid and 1 mM 
EDTA.

Western immunoblot analysis - To make cytosolic extracts, GH4 cells were 
harvested in a lysis buffer consisting of 50 mM HEPES, pH 7.5, 1 % Triton X-

100, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 10% glycerol, 1 mM Na3VO4, 
50 mM Na4P2O7, 1 mM NaF, 1 mM [4-(2-aminoethyl)-benzenesulfonylfluoride, 
HCl], and 10 µg/ ml aprotinin. For western blot of nuclear extracts, the cells were 
lysed in lysis buffer as above and the nuclei were collected by centrifugation. 
The nuclei were then extracted with 0.4 M KCl as described above for gel 
 mobility shift experiments. Protein was determined using the Bradford reagent 
(Bio Rad). Lysates were then mixed with Laemmli sample buffer and analyzed 
by SDS-polyacrylamide gel electrophoresis using 10 % gels. The proteins were 
then transferred to nitrocellulose membranes (Micron Separations). Immunoblot 
analysis was performed with antibodies to JNK/SAP, phospho -JNK/SAP, c-Jun
and phospho c-Jun from Cell Signaling Technology. Horseradish peroxidase coupled secondary antibody (Upstate) and Supersignal West Pico (Pierce) were used to visualize antibody-antigen complexes on BioMax MR film (Kodak).

RESULTS

*Insulin and oxidative stress activate the PAI-1 promoter additively* – The high levels of circulating PAI-1 found in diabetics could result from insulin stimulation of PAI-1 production as suggested previously (14,28). High levels of oxidative stress associated with diabetes might also contribute to maintenance of high PAI-1 (29). Therefore, we determined the effect of oxidative stress caused by tertbutylhydroquinone (TBHQ) on PAI-1 mRNA levels. GH4 cells express measurable PAI-1 mRNA that was increased by TBHQ treatment with a maximal response at 24 h (fig. 1A). Treatment with insulin also increased PAI-1 mRNA production and the combined treatment with both TBHQ and insulin increased in PAI-1 mRNA additively (fig. 1B). This suggested that oxidative stress and insulin acted independently to increase PAI-1 levels. The antioxidant glutathione was used to confirm that increased PAI-1 mRNA levels in TBHQ-treated cells resulted from oxidative stress. Glutathione completely prevented the increase in PAI-1 mRNA seen in TBHQ-treated cells (fig. 1B).

To determine whether the effect of oxidative stress was mediated by an increase in PAI-1 promoter activity, GH4 cells were electroporated with a PAI-1-CAT reporter plasmid and then treated with oxidants. Both TBHQ and H$_2$O$_2$ induced a 3-fold increase in PAI-1-Luc expression (Fig. 2A) in this assay. The
combined effect of insulin and oxidative stress on PAI-1 promoter function is shown in fig. 2B. Treatment of GH4 cells with both insulin and TBHQ increased PAI-1 transcription additively (Fig. 2B). These increases are fully prevented by incubation with antioxidants. Reduced glutathione blocked the effects of TBHQ but not the effect of insulin on PAI-1 gene transcription (Fig. 2C).

The AP-1 response element of the PAI-1 promoter mediated effects of oxidants - An analysis of the PAI-1 promoter was done to determine the response element that mediated the effect of oxidants. This would indicate the transcription factor family that mediated the response and allow us to examine signaling by the oxidants. Deletion of the promoter to -116 had no effect on oxidant-increased PAI-1 gene expression (Fig. 3A). This indicated that the oxidant response element was in the region -116/-6. This region contains elements for the Ets-related transcription factors, for SP-1, for FKHR-related factors, and for AP-1. Since AP-1 sites were shown to be oxidant responsive (30), a mutant of the AP-1 element of the PAI-1 promoter was tested and found to be unresponsive to oxidants while it retained insulin responsiveness (Fig. 3B). For comparison, a mutant of the insulin response element (14) remained oxidant sensitive while it lost insulin responsiveness as previously shown. Finally, a 3X-AP-1-Luciferase reporter plasmid was oxidant sensitive but unresponsive to insulin (Fig. 3B).

Oxidants increased binding to the AP-1 response element - To determine if oxidants increased the binding of transcription factors to the AP-1 element, nuclear extracts from untreated and oxidant treated cells were used in gel-
mobility shift experiments. Two retarded complexes were observed using nuclear extracts from GH4 cells (Fig. 4). The fastest migrating complex was non-specific since it was efficiently competed with either a 100-fold excess of specific or nonspecific competitor. The more slowly migrating complex was specific for the AP-1 response element since it was completely eliminated using a 100-fold excess of homologous competitor but it was only slightly affected by an oligonucleotide containing a mutation in the AP-1 response element.

**Oxidants increased c-Jun mRNA levels** - Agents that increase oxidative stress could act to increase the production of c-Fos and/or c-Jun or they could act to increase phosphorylation of c-Jun or both. The levels of c-Fos and c-Jun mRNA were measured by RT-PCR. TBHQ induced a transient increase in c-Jun mRNA levels that was maximal by 2 h and declined by 4 h (Fig. 5). In contrast, no change was noted in the level of c-Fos mRNA.

**Oxidants increased accumulation of phospho-c-Jun in the nucleus** - c-Jun was shown to be phosphorylated by MAP kinases (31). These ser/thr kinases are activated in the cytoplasm by phosphorylation on both ser/thr and tyr. They translocate to the nucleus after activation and phosphorylate transcription factors such as c-Jun in the nucleus. To determine if oxidants increased c-Jun phosphorylation, GH4 cells were treated with TBHQ for various times and cytosolic and nuclear extracts were prepared. The extracts were resolved on SDS-PAGE followed by blotting to nitrocellulose. The blots were probed with antibodies to c-Jun and phospho-c-Jun to determine if oxidative stress increased
c-Jun phosphorylation (fig. 6). Levels of phospho-c-Jun increased rapidly in both the cytosolic and nuclear fraction. The cytosolic phospho-c-Jun declined after 1 h and was not detectable at 4 h. The phospho-c-Jun level in the nucleus remained relatively constant between 8 min. and 4 h. Levels of total c-Jun in the nucleus also increased in TBHQ treated cells, but then stayed relatively constant while c-Jun in the cytoplasm was relatively constant throughout the experiment. Thus, oxidants rapidly increase the level of nuclear phospho-c-Jun.

Antioxidant blocked TBHQ-increased nuclear phospho-c-Jun accumulation - Since reduced glutathione prevented TBHQ increased PAI-1 transcription, it seemed likely that it might affect the TBHQ mediated increase in nuclear phospho-c-Jun. GH4 cells were treated with 15 mM reduced glutathione for 1 h followed by TBHQ for 2 or 4 h. Control cells received TBHQ for 2 or 4 h or were untreated. Nuclear extracts were prepared, resolved on SDS-PAGE and blotted to nitrocellulose. Western analysis with andti-phospho-c-Jun and anti-c-Jun antibodies revealed that nuclear phospho-c-Jun was increased by TBHQ in control cells (fig. 7). This confirmed our previous finding (fig. 5). In contrast, total and phospho-c-Jun decreased in nuclear extracts of cells treated with reduced glutathione.

JNK/SAP activity was increased by oxidant - The increased-phospho-c-Jun in the nucleus after treatment with TBHQ suggested that c-Jun might be phosphorylated by Jun N-terminal kinase/stress activated kinase (JNK/SAP). This was directly tested using the inhibitor SP600125 (JNKII, Calbiochem) (32).
GH4 cells that were electroporated with the PAI-1 reporter plasmid were treated for two hours with inhibitors of MAP kinases and then exposed to TBHQ for 24 h. The PD98059 that inhibits Erk1/2 and SB203580 that inhibits p38 stress activated kinase were without effect (fig. 8A). The AKT inhibitor SH5, the PI 3-kinase inhibitor LY294002, the mTOR inhibitor rapamycin, and the PKA inhibitors KT5720 and H89 also failed to block TBHQ-induced PAI-1 gene transcription (fig. 8A). Treatment with 10 μM SP600125 reduced TBHQ increased PAI-1 luciferase activity by 75%. This suggested that part or all of the effect of TBHQ was mediated through JNK/SAP-mediated phosphorylation of c-Jun. The effect of insulin on PAI-1-Luciferase activity was not affected by SP600125 (fig. 8B).

If JNK/SAP mediated phosphorylation is important for TBHQ-increased transcription, the activity of JNK/SAP should be increased by oxidants. Western analysis of cytoplasmic and nuclear JNK/SAP and phospho JNK/SAP confirmed this. Treatment with TBHQ resulted in rapid increase in phosphorylated JNK/SAP that later declined to basal levels while the level of total JNK/SAP, at first constant, declined at 2 and 4 h (fig. 9). This suggested that JNK/SAP was activated by oxidants and that activated JNK/SAP then translocated to the nucleus. Nuclear phospho JNK/SAP was increased rapidly from undetectable levels in untreated cells to a maximum at 30 min. and then declined to undetectable levels by 2 h. These changes are identical with those seen in cytosolic JNK/SAP. Total nuclear JNK/SAP remained constant until a slight decline after 4 h. with TBHQ. These experiments combined to suggest that
TBHQ initiated a signaling cascade terminating in the phosphorylation of c-Jun by JNK/SAP that increased PAI-1 gene expression.

**DISCUSSION**

The experiments reported here demonstrated that oxidative stress activated a signaling cascade resulting in increased AP-1 binding (fig. 3) secondary to activation of JNK/SAP (fig. 7) and phosphorylation of c-Jun (fig. 5). This caused the 3- to 4-fold increase in PAI-1 promoter activity in response to chemical mediators of oxidative stress. The effect of oxidative stress was additive with the effect of insulin under these conditions. This has important implications for the understanding of diabetic complications.

A large number of studies implicated AP-1 in mediating increased transcription in response to oxidants (18,20,30,33-35). Our experiments demonstrated that the AP-1 response element of the PAI-1 promoter was necessary for oxidant increased PAI-1 transcription (fig. 2) and that oxidants increased binding to the AP-1 response element of the PAI-1 promoter (fig. 3). Further, oxidants increased phospho-c-Jun (fig. 5) and c-Jun mRNA (fig 4), crucial components of the AP-1 complex. All of these data support a model in which oxidants activate PAI-1 through increased AP-1 binding to the AP-1 response element of the PAI-1 promoter. Several reports suggested that glucose-increased PAI-1 gene transcription was dependent on activation of AP-1 (36,37) although neither of these did the precise mutagenesis of the AP-1 response element needed to confirm this hypothesis. These studies were
contradicted by a previous study that localized glucose activated PAI-1 transcription to the SP-1 sites in the promoter (38). It would be interesting, however, to determine if high glucose activation of PAI-1 in these cells could be inhibited by antioxidants. This would suggest that glucose could increase the oxidative state of the cells to activate PAI-1 transcription through AP-1 and establish a direct mechanism for activation of PAI-1 in diabetes.

Many protein kinase-signaling pathways were shown to activate AP-1. These included Erk (39), p38 (40-42), JNK/SAPK (43), B/Akt (44) and p90RSK (45). The JNK/SAP inhibitor SP600125 blocked TBHQ-increased PAI-1 expression while other inhibitors had no effect (fig.8 and data not shown) and TBHQ increased the activity of JNK/SAP (fig. 9). This suggested that the increased phosphorylation of nuclear c-Jun that we observed (fig.7) was the result of phosphorylation by JNK/SAP. These studies found increases in both nuclear c-Jun phosphorylation and in cJun mRNA levels in response to TBHQ. It is unclear how each of these contributes to increased PAI-1 transcription. The stimulation of PAI-1 mRNA levels is a long-term process that peaks at approximately 24 h. This makes it possible that the first phase response is mediated by increased c-Jun phosphorylation while the continued response results from elevated c-Jun protein. It is not possible to come to any firm conclusions in this regard without a more thorough analysis of potential components of the AP-1 complex in GH cells (ie, Fos, Fra, JunB, etc.) and an understanding of the half-life of the mRNA and proteins.
Oxidative stress mediated activation of JNK/SAP activated transcription of some genes (46) while inhibiting the transcription of others (47). Thus, oxidant-sensitive activation of JNK/SAP probably mediates much of the cellular response to oxidative stress by turning on genes responsive to AP-1 and NFκB (35,46) while it turns off genes responsive to other transcription factors, e.g. PDX (47).

Signaling by oxidation products ended in the phosphorylation of c-Jun by activated JNK/SAP. It is unclear how this pathway is activated. JNK/SAP is a member of the MAP kinase family of kinases that is conserved from yeast to mammals. MAP kinases are activated by small GTP binding proteins through a phosphorylation cascade that may involve activation of: 1, a MAP Kinase Kinase Kinase Kinase, 2, a MAP Kinase Kinase Kinase Kinase, and 3, a MAP Kinase Kinase. Several kinases can act at each level of this cascade and some may be GTP binding protein-dependent (Ras, Rac, CDC42) while others are not. Thus at the MAP Kinase kinase kinase kinase level both germinal center kinases (Rac/cdc42 independent) and p21 associated kinase (Rac/cdc42 dependent) were shown to activate JNK/SAP. Raf (Rac/cdc42 independent), mixed lineage kinases (Rac/cdc42 dependent and independent), and MEKK (Rac/cdc42 dependent) activated JNK/SAP at the MAPKinase kinase kinase level. Finally, MKK4/MKK7 activation by phosphorylation results in increased phosphorylation of JNK/SAP. Stress, injury and cytokines all activate the pathways leading to activation of JNK/SAP.
Oxidative stress-activated protein kinase C (PKC) represents another possible way to activate JNK/SAP and AP-1. The AP-1 site of the PAI-1 promoter was phorbol ester responsive (48) and ionizing radiation activated JNK/SAP-1 through PKC and MKK7 (49). Oxidants also activated various phospholipases (50-53) although it is unclear how this occurred. Thus, it is possible that oxidants activate phospholipase that in turn stimulates an isoform of PKC, MKK7 and JNK/SAP. We are currently exploring this possibility.

Oxidants have been reported to stimulate apoptosis in a number of cell types (18,54). This was attributed to activation of c-Jun/AP-1 in at least one case (18) although others found both NF-κB and AP-1 to be necessary (54). We did not see evidence for increased apoptosis. This was probably not due to the length of the incubation with oxidants since apoptosis was observed within 30 min. It might depend on the cell-type or dose that was used since the agents used here caused cell death in other cell types at higher concentrations.

Some of the effects of oxidative stress in vivo may be due to activation of pro inflammatory cytokines. The relationship between oxidants and cytokines is complex. Oxidants both stimulated the production of cytokines (55,56) and mediated effects of cytokines (57). Both oxidants and cytokines were reported to signal through PKC isoforms (56,58-60). IL-1β and phorbol esters stimulated PAI-1 expression through the same AP-1 response element that responded to oxidant stress (48). Thus, oxidative stress and cytokines may establish a positive feedback loop that could markedly increase transcription of the PAI-1
promoter in either type I or type II diabetes. This could be an autocrine loop since oxidants activated cytokine production in HepG2 and liver parenchymal cells (56). Finally, oxidant activation of other growth factors, e.g. IGF-I, is also possible.

PAI-1 has been implicated in many of the complications of diabetes. It plays a role in atherosclerosis by its inhibition of fibrinolysis (61). It plays a role in wound healing through its effects on keratinocyte cell migration (62). It is a factor promoting glomerulosclerosis and tubulointerstitial fibrosis making it an important candidate in diabetic nephropathy (63). It is necessary for peripheral nerve regeneration establishing a link to peripheral neuropathy (64). It is overexpressed in diabetic retina and may play an important role in excess vascularization (65). Thus, it is important to limit PAI-1 production in diabetes. We demonstrated that hyperinsulinemia and oxidative stress act additively to activate transcription of this gene by stimulating tandem elements in the promoter. Cytokines are also likely act through this stress element. Thus, the three factors that are most likely responsible for the inappropriate regulation of PAI-1 in diabetes act at this composite element. A concerted effort to block this element would likely yield important improvements in PAI-1 levels and diabetic complications. But selective blockade of this element using pharmacological inhibitors or transcription factor decoys could prove difficult. The realization that oxidative stress is responsible for a major proportion of the increase is significant since this could be prevented by antioxidant therapies. Which of these would be
effective is difficult to predict, but the response of the PAI-1 promoter could be
used to screen for agents that would be most effective for this purpose.

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ABBREVIATIONS USED: Plasminogen Activator Inhibitor-1, PAI-1; tertbutylhydroquinone, TBHQ; reverse transcriptase-polymerase chain reaction, RT-PCR: human insulin receptor, HIR

FIGURE LEGENDS

Figure 1 – PAI-1 mRNA production in response to TBHQ and insulin – A) GH4 cells were treated with 100 µM TBHQ for the times indicated. The cells were harvested, mRNA was prepared, and RT-PCR of PAI-1 mRNA was conducted as described in Materials and Methods. The RT-PCR products were separated by agarose gel electrophoresis and visualized using a Fluroimager (Amersham). A typical image is shown. B) GH4 cells were treated with 100 µM TBHQ or 1 µg/mL of insulin or both insulin and TBHQ. Some cultures were also treated with 10 mM glutathione before TBHQ treatment. The mRNA was prepared and RT-PCR of PAI-1 was performed. A Fluoroimager image of two typical experiments is shown.

Figure 2 – Activation of the PAI-1 promoter by oxidative stress – GH4 cells were electroporated with 10 µg of the PAI-1-luciferase and 1 µg of RSV-β-Gal. After 24 hours, the medium was exchanged and various treatments were applied. The plates were harvested 48 h after electroporation by washing 3 times with normal saline and freezing. The relative light units/100 µg protein in control and experimental cultures was determined, adjusted for β-galactosidase expression, and the luciferase activity from the treated cells was compared with the control level to determine the fold-stimulation (Fold-Control). The results ± S.E.M. are
from three separate experiments done in triplicate. **A.** Vehicle, 100 µM TBHQ or 100 µM H₂O₂ was added for 24 h before harvest. **B.** Vehicle, 100 µM TBHQ, 1 µg/ml insulin or both TBHQ and insulin were added 24 h before harvest. **C.** One-half of the cells were treated with 15 mM reduced glutathione for 1 h and half of the cells were left untreated as controls. Vehicle (DMSO), 100 µM TBHQ, 1 µg/ml insulin or both insulin and TBHQ were then added for 24 h.

Figure 3 – Mutation analysis of the PAI-1 promoter – GH4 cells were electroporated with 10 µg of the PAI-1-luciferase reporter plasmid indicated in the figure and 1 µg of RSV-β-Gal. **A.** GH4 cells transfected with 5’ deletion mutants of the PAI-1 promoter. **B.** GH4 cells transfected with point mutants of the PAI-1 promoter or with a 3xAP1-luciferase reporter plasmid. After 24 hours, the medium was exchanged and TBHQ or vehicle was added to the appropriate cultures. The plates were harvested 48 h after electroporation by washing twice with normal saline and freezing. The average relative light units/100 µg protein in control and insulin-treated cultures was determined, adjusted for β-galactosidase expression, and the luciferase activity from cells incubated with hormones were compared with control levels to determine the fold-stimulation (Fold-Control). The results ± S.E.M. are from three separate experiments done in duplicate.

Figure 4 - Binding to the AP-1 response element – A ³²P labeled oligonucleotide to the AP-1 response element was incubated with nuclear extracts from control cells (lanes 1, 4, and 5) or cells incubated with TBHQ for 2 h (lane 2) or 4 h (lane 3). The incubation in lane 4 included unlabeled AP-1 response element and the
incubation in lane 5 contained unlabeled mutant AP-1 response element at a 100-fold excess over the labeled oligonucleotide. Lane 6 contained labeled DNA alone.

Figure 5 - c-Jun mRNA production is increased by TBHQ - GH4 cells were incubated with TBHQ for the times indicated. They were harvested and mRNA was prepared as described in Experimental Procedures. The amount of mRNA for use in semi-quantitative RT-PCR was standardized to the amount of glyceraldehyde phosphate dehydrogenase mRNA in each sample. (top) Equal amounts of mRNA were used in RT-PCR reactions with primers to c-Fos or c-Jun. The PCR products were resolved on 1% agarose gel electrophoresis using Syber Green® (Molecular Probes, Eugene OR) to stain the DNA. The stained product was visualized using a Fluoroimager (Amersham) and quantitated using Image Quant software (Amersham). (Bottom) A graph of the average c-Jun mRNA (± S.E.M.) from three separate experiments.

Figure 6 - c-Jun phosphorylation in response to TBHQ - GH4 cells were incubated with TBHQ for the times indicated. Cell lysates and nuclear extracts were prepared and separated by SDS-PAGE. The gels were blotted to nitrocellulose and probed with antibodies to c-Jun and phospho-c-Jun as indicated. Top. Cytoplasmic cJun and phospho c-Jun. Bottom. Nuclear cJun and phospho c-Jun.

Figure 7 - Phospho-c-Jun accumulation in GH4 cells treated with reduced glutathione - GH4 cells were incubated with 15 mM reduced glutathione or left
untreated as controls. After 1 h, TBHQ was added for 2 or 4 h. Nuclear extracts were prepared, resolved on SDS-PAGE, blotted to nitrocellulose and western blotted with anti-c-Jun (top) and anti-phospho-c-Jun (bottom).

Figure 8 – SP600125 (JNKII) inhibits TBHQ-increased PAI-1 expression – GH4 cells were electroporated with 10 µg of the PAI-1-luciferase and 1 µg of RSV-[-Gal. A) After 24 hours, the medium was exchanged and inhibitors were added. The concentrations were: SP600125, 10 µM, LY294002, 50 µM, PD98059, 40 µM, SB203580, 10 µM, rapamycin, 1 µM, H89, 10 µM, KT5720, 10 µM and SH5, 10 µM. TBHQ was added 2 h later to half of the wells of each condition. The incubation was continued for an additional 24 h and luciferase activity was determined. Fold-control was determined as described above. The results ± S.E.M. are from three separate experiments done in triplicate.

B) After 24 hours, the medium was exchanged and 10 µM SP600125 was added to half of the wells. TBHQ, insulin, or TBHQ and insulin were added two hours later as indicated. The incubation was continued for an additional 24 h and luciferase activity was determined. The adjusted light units were compared to the untreated cells (DMSO-control) to determine fold-basal expression. The results ± S.E.M. are from three separate experiments done in triplicate.

Figure 9 - TBHQ activation of JNK/SAP - GH4 cells were incubated with TBHQ for the times indicated. Cell lysates and nuclear extracts were prepared and separated by SDS-PAGE. The gels were blotted to nitrocellulose and probed with antibodies to JNK/SAP and phospho-JNK/SAP as indicated. Top.
Cytoplasmic JNK/SAP and phospho JNK/SAP. Bottom. Nuclear JNK/SAP and phospho JNK/SAP.
Figure 1

A

Hours with TBHQ

0  6  12  18  24  PAI-1

B

Control  TBHQ  Insulin  Insulin & TBHQ  GSH  GSH & TBHQ

Experiment 1

Experiment 2

1  2  3  4  5  6  PAI-1
Figure 2

A

B

C

Vehicle

N-Acetylcysteine

Glutathione

Reduced

Luciferase Activity: Fold-Control

Treatment

Vehicle H2O2 tBHQ

Insulin

Insulin + tBHQ

Luciferase Activity: Fold-Control

Treatment

Vehicle tBHQ

H2O2

L

Luciferase Activity: Fold-Control

Treatment

Vehicle N-Acetylcyesteine Glutathione Reduced

Luciferase Activity: Fold-Control

Treatment

vehicle

TBHQ

Insulin

TBHQ + Insulin

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Figure 3

(A) Luciferase Activity: Fold-Control

-246/-7 wt  -117-7 wt

Reporter Plasmid

(B) Luciferase Activity: Fold-Control

-247/-7 AP1Mut

-246/-7 IREmut

3X AP1

Treatment

- tBHQ
- Insulin
- tBHQ + Insulin
Figure 4

Nuclear Extract

100-Fold AP-1

No Inhibitor

100-Fold AP-1

AP-1

Non-Specific

Free DNA

1 2 3 4 5 6

None
Fos and Jun mRNA Levels in Tert Butyl Hydroquinone Treated Cells

Relative units

THBQ Incubation (Minutes)

0 60 120 180 240

0 20 40 60 80 100 120 140

Relative units

THBQ Incubation (Minutes)

0 60 120 180 240

0 20 40 60 80 100 120 140

Figure 5
Phospho-c-Jun is Increased by Tert Butyl Hydroquinone

Figure 6
GSH prevents tBHQ-increased Phospho-c-Jun

Figure 6
Figure 9

Phospho-JNK is Increased by Tert Butyl Hydroquinone

A) Cytoplasmic
B) Nuclear
Oxidative stress activates the plasminogen activator inhibitor type 1 (PAI-1) promoter through an AP-1 response element and cooperates with insulin for additive effects on PAI-1 transcription
Anthony I. Vulin and Frederick M. Stanley

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