Purification and Identification of Secreted Oxidative Stress-induced Factors from Vascular Smooth Muscle Cells*

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Reactive oxygen species have been implicated in the pathogenesis of atherosclerosis and hypertension, in part by promoting vascular smooth muscle cell (VSMC) growth. We have previously shown that LY83583, a generator of O2-, activated extracellular signal-regulated kinases (ERK1/2) with early (10 min) and late (2 h) peaks and stimulated VSMC growth. To investigate whether secreted oxidative stress-induced factors (termed SOXF) from VSMC were responsible for late ERK1/2 activation in response to LY83583, we purified putative SOXF proteins from conditioned medium (2 h of LY83583 exposure) by sequential chromatography based on activation of ERK1/2. Proteins identified by capillary chromatography, electrospray ionization tandem mass spectrometry, and data base searching included heat shock protein 90-α (HSP90-α) and cyclophilin B. Western blot analysis of conditioned medium showed specific secretion of HSP90-α but not HSP90-β. Immunodepletion of HSP90-α from conditioned medium significantly inhibited conditioned medium-induced ERK1/2 activation. Human recombinant HSP90-α reproduced the effect of conditioned medium on ERK1/2 activation. These results show that brief oxidative stress causes sustained release of protein factors from VSMC that can stimulate ERK1/2. These factors may be important mediators for the effects of reactive oxygen species on vascular function.

Oxidative stress has become recognized as an important stimulus for the vessel wall. Increasing evidence suggests that increased generation of reactive oxygen species (ROS) is pathogenic for vascular diseases including hypertension, atherosclerosis, and the response to injury (1). The increase in ROS formation may occur by changes in neurohumoral state (activation of the renin angiotensin system), hemodynamic parameters (hypertension, increase stretch), and cell-cell interactions (decreased nitric oxide formation by endothelium or increased breakdown of cyclic GMP by VSMC). Recent studies have provided mechanistic insight into mediators for increased ROS with a primary focus on induction and/or activation of NAD(P)H oxidase and changes in glutathione biosynthesis (1, 2). However, there has been a relative paucity of investigation into the adaptive responses by which the vessel wall attempts to compensate for the increase in ROS.

One logical mechanism for endothelial cells and VSMC to respond to ROS would be to produce autocrine/paracrine signals that enhanced cell survival or stimulated pathways that protected cells from the damaging effects of ROS. VSMC are particularly likely to secrete protective factors that also promote cell survival based on previous studies that demonstrate secretion of a number of growth factors from VSMC in response to various stimuli. These growth factors include adrenomedulin, endothelin, epiregulin, FGF, Gas6, PDGF, and TGF-β (3–8).

The mitogen-activated protein kinases (MAPKs) respond to diverse stimuli, including growth factors and reactive oxygen species, and transduce signals from the cell membrane to the nucleus (9, 10). The extracellular signal-regulated kinases (ERK1/2, also termed p42/44MAPK), are one of the subfamilies of MAPKs and play pivotal roles in several cell functions including cell growth, transformation, and differentiation, as well as cell survival after stress (11–13).

In a previous study we observed that stimulation of VSMC with the naphthoquinolinedione LY83583 to generate O2- resulted in a biphasic activation of ERK1/2. An initial stimulation occurred with peak at 5–10 min, and a second peak occurred at 120 min. Elucidation of the events responsible for the first peak demonstrated a dependence on protein kinase C and calcium (14). Because the second peak occurred after sufficient time for de novo protein synthesis and secretion to have occurred we investigated the role of secreted autocrine factors. Using conditioned medium we purified activities by sequential column chromatography that stimulated ERK1/2 in VSMC. These activities, termed SOXF for secreted oxidative stress-induced factors, eluted at 80–100, 45–65, and 20–30 kDa on S200 gel filtration. In the present study we identify two factors (heat shock protein 90-α and cyclophilin B) and suggest that...
these are potentially important physiological mediators of the vessel wall response to ROS.

### MATERIALS AND METHODS

#### Cell Culture—VSMC
VSMC were isolated from 200–250-g male Harlan Sprague-Dawley rats and maintained in 10% calf serum/Dulbecco’s modified Eagle’s medium as described previously (14). Passages 5 to 14 VSMC at 70–90% confluence in 150-mm or 35-mm dishes were growth-arrested by incubation in 0.1% calf serum/Dulbecco’s modified Eagle’s medium for 48 h prior to use.

#### Measurement of O2 Production—Chemiluminescence of lucigenin (bis-N-methylacridinium nitrate) was used to measure O2 production with photon emission detected by a scintillation counter (LS 7000, Beckman Instruments, Inc.) in out-of-coincidence mode with a single active photomultiplier tube. A buffer blank was subtracted from each reading before conversion of the data (14).

#### Determination of Cell Viability—A modified MTT assay that measures mitochondrial function was used to determine cell viability (15). VSMC (1 × 104 cells/ml) were grown in 96-well plates for 24 h. After incubation with agonists for 2 h, cells were treated with MTT (0.5 mg/ml) for 4 h at 37 °C. The cell culture medium was removed, and cells were lysed by addition of 100 μl of isomyl alcohol. The metabolized MTT was measured in an enzyme-linked immunosorbent assay reader at 570–690 nm.

#### Preparation of Conditioned Medium—Growth-arrested VSMC in 150-mm dishes were washed three times with HBSS (containing 130 mM NaCl, 5 mM KCl, 1.5 mM CaCl2, 1.0 mM MgCl2, 20 mM HEPES, pH 7.4) and equilibrated in HBSS for 1 h. Cells were then exposed to 1 μM LY83583 or HBSS only for 2 h. Conditioned medium from LY83583-stimulated cells or control medium from HBSS-incubated cells was then collected, and protease inhibitors were added (1 mM benzamidine, 1 ng/ml pepstatin A, and 0.1% β-mercaptoethanol). The medium was centrifuged for 10 min at 800 × g to remove cell debris and stored at −80 °C.

#### Purification Scheme—For routine purification, 1000 ml of LY83583 conditioned medium was collected, dialyzed against buffer A (20 mM phosphate-buffered saline, pH 7.0, 1 mM benzamidine, 1 mg/ml pepstatin A, 1 mM dithiothreitol, and 5% glycerol) and applied to a SP-Sepharose column (5 ml of HiTrap SP, Amersham Pharmacia Biotech) equilibrated with the same buffer. Fractions were eluted by buffer A with stepwise 0.5 M increases in [NaCl], and 50-μl aliquots of each fraction were assayed for ERK1/2 stimulating activity. The active fractions were pooled, dialyzed against buffer B (10 mM phosphate-buffered saline, pH 7.0, 1 mM benzamidine, 1 mg/ml pepstatin A, 1 mM dithiothreitol, and 5% glycerol), and subjected to heparin-Sepharose Chromatography (5 ml of HiTrap heparin, Amersham Pharmacia Biotech) equilibrated with buffer B. The column was eluted with a stepwise gradient of 0.1–2.0 M NaCl in the same buffer. 25-μl aliquots of each fraction were used to measure ERK1/2 stimulating activity. The active fractions were pooled, dialyzed against buffer C (50 mM phosphate-buffered saline, pH 7.0, 1 mM benzamidine, 1 mg/ml pepstatin A, 1 mM dithiothreitol, and 5% glycerol), adjusted to a final concentration of 1.0 mM ammonium sulfate, and subjected to heparin-Sepharose chromatography (1 ml, Amersham Pharmacia Biotech). The column was developed with a stepwise descending gradient of ammonium sulfate in buffer C. ERK1/2 stimulating activity eluted as a single peak at 0.15 M ammonium sulfate. The active fraction was concentrated to 0.3 ml with Centricon 3 and placed on a Sephacryl S200 gel filtration column (1 × 90 cm, Amersham Pharmacia Biotech). The column was equilibrated with buffer D (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.02% NaN3) and eluted at a flow rate of 0.2 ml/min. Fractions (2 ml) were collected, and 50-μl aliquots were used for ERK1/2 kinase assay.

#### Protein Kinase Analysis—Cells grown in 35-mm dishes were growth-arrested for 48 h in 0.1% serum and stimulated with 2 ml of medium. For harvest, cells were washed with phosphate-buffered saline and scraped into 0.2 ml of lysis buffer (10 mM Tris, pH 7.5, 5 mM MgCl2, 1 mM EDTA, 25 mM NaF), prepared with fresh 20 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride. Cell lysates were prepared by sonication for 1 s and centrifugation for 30 min at 15,000 × g. Protein concentration in the supernatant was determined by Bradford protein assay, and the samples were stored at 80 °C. Lysates were analyzed as described previously for ERK1/2 activity by Western blotting with phosphospecific ERK1/2 antibody (16).

#### Protein Identification by Capillary Chromatography, Electrospray Ionization Tandem Mass Spectrometry—Fraction III from phenyl-Sepharose chromatography was separated by 12% PAGE. Proteins were stained with 0.1% Coomassie Brilliant Blue 250 in 40% methanol and 1% acetic acid and destained in 50% methanol. Protein bands were excised, chopped into 1-mm2 pieces, and subjected to in-gel digestion with trypsin (17). Proteins were identified by collecting sequence information from the resulting peptides by micropipillary LC-MS/MS techniques (18). Briefly, peptides eluting from the capillary column were automatically fragmented by collision-induced dissociation in a triple quadrupole mass spectrometer (TSQ 7000, Finnigan-Mat, San Jose, CA), and the resulting sequence information was recorded in a tandem (MS/MS) or collision-induced dissociation mass spectrum. Collision-induced dissociation spectra were then computer-searched using the Sequest algorithm (19) against the OWL non-redundant data base as well as the EST data bases. Protein identification was unambiguous because multiple peptides from each protein were matched by the data base searches.

#### Immuno depletion—Concentrated conditioned medium was incubated with rabbit anti-HSP90-α polyclonal antibody (1:100 dilution) (StressGen Biotechnologies Corp.) or an equal amount of rabbit IgG for 22 h and then incubated with protein A-agarose (Life Technologies, Inc.) for 2 h on a roller system at 4 °C. The supernatants and control medium were used to stimulate growth-arrested rat VSMC in 35-mm dishes, and ERK1/2 activity was analyzed by Western blot.

#### Statistical Analysis—Data are shown as mean ± S.E. Differences were analyzed with one-way analysis of variance (ANOVA), and post-hoc analysis was performed using Bonferroni/Dunn. Values of p < 0.05 were considered statistically significant.

### RESULTS

**Secreted Factors Are Involved in Regulation of ERK1/2 Activation by Oxidative Stress**—To generate oxidative stress...
VSMC were exposed to LY83583, which generates $O_2^\cdot$ (14). Production of $O_2^\cdot$ in VSMC exposed to 1 $\mu$M LY83583 was measured by lucigenin chemiluminescence as described previously (14). LY83583-induced generation of $O_2^\cdot$ peaked at 15 min and returned to baseline by 120 min (Fig. 1A). Tiron (10 mM), a membrane-permeant nonenzymatic $O_2^\cdot$ scavenger, completely abolished LY83583-induced $O_2^\cdot$ (Fig. 1A). In contrast, catalase (10 units/ml), an extracellular scavenger of $H_2O_2$, had no significant effect on LY83583-induced $O_2^\cdot$ (Fig. 1A).

Previously we showed that brief exposure of VSMC to LY83583 stimulated ERK1/2 activity with peak at 10 min and return to baseline at 30 min (14). In this new series of experiments, cells were studied for 240 min after LY83583 exposure. A second peak of ERK1/2 activation was apparent at 120 min, a time when $O_2^\cdot$ production was minimal. LY83583 (10 nM) prior to exposure to LY83583 (Fig. 1B). ERK1/2 activity at 120 min was greater than activity at 10 min and was nearly equivalent to that observed with PDGF or with xanthine plus xanthine oxidase.

The results were observed when conditioned medium was prepared from cells treated with 200 $\mu$M $H_2O_2$, 50 $\mu$M menadione, or 100 $\mu$M xanthine/xanthine oxidase (Fig. 1C). A Trypsin-sensitive Secreted Oxidative Stress-induced Factor (SOXF) Is Released in Response to LY83583—To verify that the second peak of ERK1/2 activity was not related to cell death induced by LY83583, cell viability after a 120-min treatment with LY83583 was analyzed by a modified MTT assay. There was no significant decrease in cell viability when cells were exposed to <10 $\mu$M LY83583 for 120 min (Fig. 2A). In addition, there was no noticeable change of morphology in VSMC after treatment with LY83583 for 120 min (not shown). Finally, LY83583 did not cause a significant increase in release of lactate dehydrogenase into conditioned medium (not shown).

These results suggest that early activation of ERK1/2 is due to increased generation of $O_2^\cdot$, whereas the later activation of ERK1/2, which occurs at a time when $O_2^\cdot$ production is low, may be due to stimulation of autocrine mechanisms such as release of factors into the medium.

To identify the presence of SOXF from VSMC stimulated by LY83583, the ability of conditioned medium to stimulate ERK1/2 was assayed. Conditioned medium was prepared as described in Fig. 1 and then transferred to growth-arrested VSMC for 10 min. ERK1/2 activity, measured with a phospho-ERK1/2 specific antibody, demonstrated a significant increase with conditioned medium from cells treated with LY83583, but not control (Fig. 2B). The activity was sensitive to trypsin (5 $\mu$g/ml for 30 min) and was inhibited by heating to 100 °C (Fig. 2B). Pretreatment with actinomycin D or cycloheximide at concentrations that inhibit RNA and protein synthesis had no significant effect on the ability of conditioned medium to stimulate ERK1/2 (not shown).

To prove that formation of SOXF required generation of $O_2^\cdot$ and did not require generation of $O_2^\cdot$, VSMC were pretreated with the metal chelating agent Tiron (10 mM) prior to exposure to LY83583. Tiron completely inhibited its production of $O_2^\cdot$ by LY83583 (Fig. 1A). Tiron pretreatment completely inhibited the appearance of ERK1/2 stimulating activity in LY83583-conditioned medium (Fig. 2C, lane 6). However, when LY83583-conditioned medium from control cells was placed on cells pretreated with Tiron, ERK1/2 activity was not inhibited (Fig. 2C, lanes 3 and 4). These findings demonstrate that the activity present in conditioned medium does not require generation of $O_2^\cdot$ to stimulate ERK1/2. Previously (14), we showed that Tiron did not inhibit activation of ERK1/2 by phorbol ester or angiotensin II, demonstrating spec-
Purification of SOXF

**TABLE I**

Summary of SOXF purification from 500 ml of conditioned medium

| Purification step         | Protein recovered | Total activity | Specific activity | Activity recovered | Purification factor |
|--------------------------|-------------------|----------------|-------------------|--------------------|--------------------|
| Conditioned medium       | µg                | units          | units/µg          | %                  | —                  |
| SP-Sepharose             | 165.0             | 45,000.0       | 2.0               | 90.0               | 100.0              |
| Heparin-Sepharose        | 16.0              | 30,000.0       | 2.0               | 60.0               | 666.7              |
| Phenyl-Sepharose         | 3.0*              | 8,400.0        | 2.8               | 16.8               | 4,000.0            |
| S200 gel filtration      | 2.0*              | 5,500.0        | 2.5               | 11.0               | 12,500.0           |

*a* One unit was defined as the amount of sample required to stimulate ERK1/2 activity to a level 100% of that observed for 1 µM LY83583 at 10 min in rat VSMC.

*b* Estimated by Coomassie stain of SDS-PAGE.

ifi. Based on these findings we conclude that LY83583 stimulates release of a factor(s) (most likely a protein) into conditioned medium that activates ERK1/2, even though the factor does not itself generate O$_2$$^\bullet$. To determine whether a few proteins comprised the majority of SOXF, we analyzed the proteins released into the medium in response to LY83583. Cells were labeled with $[^{35}S]$methionine for 4 h and then exposed to 1 µM LY83583 for 120 min. Conditioned medium was harvested and concentrated, and proteins were analyzed by 5–15% SDS-PAGE. Approximately 35 protein bands were detected by autoradiography (not shown) suggesting that multiple proteins were released in response to LY83583. We therefore sought to purify the activity, which we named SOXF for secreted oxidative stress-induced factor, on the basis of its ability to stimulate ERK1/2.

**Purification of SOXF**—To purify SOXF we used a sequential chromatographic approach that involved SP-Sepharose, heparin-Sepharose, phenyl-Sepharose, and S200 gel filtration chromatography (Fig. 3, Table I). For each step, activity was assayed for ERK1/2 stimulation by phospho-ERK1/2 Western blot analysis of proteins in the 90-kDa band revealed cyclophilin B. No immunoreactivity was detected (not shown). Neutralizing antibodies raised to these growth factors were also used to determine whether these factors contribute to SOXF activity. In comparison to goat IgG, none of the neutralizing growth factor antibodies caused any significant decrease in SOXF activity present in the heparin-Sepharose fractions (Fig. 6). Thus SOXF does not appear to be a known VSMC heparin-binding growth factor.

The heparin-Sepharose fractions were subjected to phenyl-Sepharose chromatography to purify heparin-binding proteins (such as PDGF and FGF) that may have been secreted. On heparin-Sepharose, SOXF activity eluted as a broad peak at 0.75–1.5 M NaCl (Fig. 5). Only a small amount of activity failed to bind to heparin-Sepharose (Fig. 5A, Flow through). To determine whether heparin binding growth factors were present in the 0.75–1.5 M NaCl fraction, Western blot analysis was performed on concentrated fractions with antibodies against basic FGF, HB-EGF, PDGF, and TGF-β. No immunoreactivity was detected (not shown). Neutralizing antibodies raised to these growth factors were also used to determine whether these factors contribute to SOXF activity. In comparison to goat IgG, none of the neutralizing growth factor antibodies caused any significant decrease in SOXF activity present in the heparin-Sepharose fractions (Fig. 6). Thus SOXF does not appear to be a known VSMC heparin-binding growth factor.

The heparin-Sepharose fractions were subjected to phenyl-Sepharose chromatography to separate lipids that may have been bound to proteins. On phenyl-Sepharose, SOXF activity eluted as a single peak at 0.15 M (NH$_4$)$_2$SO$_4$ (Fig. 7). Only a small amount of activity failed to bind to phenyl-Sepharose (not shown). The 0.15 M (NH$_4$)$_2$SO$_4$ fraction from phenyl-Sepharose was concentrated and subjected to 12% SDS-PAGE, followed by protein detection with Coomassie Blue (Fig. 8). The dominant protein band was identified at 90 kDa, with additional bands at 92 kDa, 56 kDa, 27 kDa, and 20 kDa.

The 0.15 M (NH$_4$)$_2$SO$_4$ fraction from phenyl-Sepharose was subjected to S200-gel filtration chromatography to determine the molecular sizes of proteins with SOXF activity. On S200 chromatography, SOXF activity eluted in three peaks at approximate molecular masses (in order of relative ERK1/2 stimulating activity) of 80–100 kDa > 20–30 kDa > 45–65 kDa (Fig. 9).

**Identification of SOXF Candidate Proteins by Mass Spectrometry**—Individual protein bands were excised from the Coomassie Blue-stained gel shown in Fig. 8. The proteins were digested by trypsin, and the resulting peptides were analyzed by an electrospray triple quadrupole mass spectrometer. The collision-induced dissociation spectra generated were used to identify the proteins from which the peptide originated by database searching using the Sequest software program. Unambiguous identification of proteins in the 90-kDa band revealed heat shock protein 90-α (HSP90-α). Unambiguous identification of proteins in the 20-kDa band revealed cyclophilin B. No proteins were identified in the 56-kDa and 27-kDa bands.

**LY83583 Stimulates Release of HSP90-α Specifically from VSMC**—To prove that proteins identified as putative SOXFs were released specifically from LY83583-treated VSMC, a conditioned medium experiment was analyzed. To prove specificity we compared the relative protein abundance of candidate SOXF proteins in the total cell lysate and in conditioned medium, before and after LY83583 stimulation. Because HSP90-α was the largest SOXF identified, and therefore least likely to be released nonspecifically, we studied its abundance. Condi-
tioned media from control and LY83583-treated cells were con-
centrated and subjected to 12% SDS-PAGE followed by West-
ern blot analysis for HSP90 (with an antibody that recognizes
HSP90-a and HSP90-b). The abundance of HSP90 was
compared with two intracellular proteins of similar molecular
sizes; PKC-z (with an antibody that also recognizes PKC-a)
and c-Raf-1 (Fig. 10). Conditioned medium from control cells
showed very low levels of HSP90-a and no detectable PKC or
c-Raf-1 (lane 1). After treatment with LY83583 for 2 h, there
was a 10-fold increase in HSP90-a present in the conditioned
medium (Fig. 10, lane 2), but no detectable HSP90-b, PKC, or
c-Raf-1. These results suggest that regulated secretion of
HSP90-a occurred in response to LY83583. To prove that the
secretion of HSP90-a was not a consequence of dramatic
changes in protein expression we also assayed the levels of
protein in total cell lysate (Fig. 10, TCL). Whereas PKC-a,
PKC-ζ, and c-Raf-1 showed no change in expression, both
HSP90-a and HSP90-b showed increased expression in re-
response to LY83583 (for HSP90-a expression included both cy-
toplasmic and secreted protein). However, the magnitude of the
increase in HSP90 protein expression was much smaller than
the change in levels of secreted protein.

Immunodepletion of HSP90-a Inhibits Conditioned Medium-
induced ERK1/2 Activation—To determine the extent to which
secreted HSP90-a contributes to ERK1/2 activation at 120 min
in response to LY83583, HSP90-a in conditioned medium was
immunodepleted with anti-HSP90-a antibody (Fig. 11). The
supernatant of conditioned medium, following immunoprecipi-
tation of HSP90-a, showed 90% decrease in immunoreactive
HSP90-a protein, whereas rabbit IgG caused no significant
depletion (Fig. 11A). Immunodepletion of HSP90-a signifi-
cantly inhibited conditioned medium-induced ERK1/2 activa-
tion (51.3 ± 12.0% decrease, p < 0.01, n = 4) in VSMC, whereas
immunodepletion with rabbit IgG had no significant effect (Fig.
11, B and C). These findings indicate that HSP90-a is a SOXF

**FIG. 5.** Heparin-Sepharose chromatography of SP-Sepharose fractions. The 0.75–1.25 M NaCl fraction from SP-Sepharose was dia-
lyzed against buffer B and applied to a heparin-Sepharose column equilibrated in buffer B. Proteins were eluted by stepwise increases in
[NaCl] in buffer B. Fractions (25 μl) were applied directly to growth-arrested VSMC for assay of ERK1/2 activity (A), which was quantified
by densitometry and expressed in arbitrary density units (B). SOXF* indicates putative SOXF-containing fraction from SP-Sepharose column.

**FIG. 6.** Effect of neutralizing antibodies on SOXF activity of heparin-Sepharose fractions. The 0.75–1.5 M NaCl fraction from heparin-Sepharose was incubated for 1 h at 37 °C with goat IgG or
neutralizing antibodies against HB-EGF, PDGF-BB, basic FGF, or
TGF-β. The treated fractions were combined with protein G-agarose,
and proteins were centrifuged for 10 min to precipitate proteins. The
supernatants were then applied directly to growth-arrested VSMC for
ERK1/2 activity. Results are representative of three experiments.

**FIG. 7.** Phenyl-Sepharose chromatography of SP-Sepharose fractions. The 0.75–1.5 M NaCl fraction from heparin-Sepharose was dia-
yzed against buffer C, adjusted to a final concentration of 1 M ammonium sulfate, and applied to a phenyl-Sepharose column equili-
brated in buffer C containing 1 M ammonium sulfate. Proteins were
eluted by stepwise decrease in [ammonium sulfate] in buffer C. Fractions (15 μl) were applied directly to growth-arrested VSMC for assay of
ERK1/2 activity (A), which was quantified by densitometry and ex-
pressed in arbitrary density units (B). SOXF* indicates putative SOXF-containing fraction from heparin-Sepharose column.

**FIG. 8.** Coomassie stain of the 0.15 M ammonium sulfate frac-
tion from phenyl-Sepharose (SOXF). Fraction III from phenyl-
Sepharose chromatography containing ~5 μg of protein was subjected
to 12% SDS-PAGE. Proteins were stained with 0.1% Coomassie Bril-
liant Blue 250 in 40% methanol and 1% acetic acid, destained in 50%
methanol. Approximate molecular masses were determined by logarith-
mic plot of migration of molecular mass markers. SOXF* indicates
putative SOXF-containing fraction from phenyl-Sepharose column.
that accounts for approximately 50% of the late-phase ERK1/2 activation stimulated by LY83583.

**Human Recombinant HSP90-α Activates ERK1/2**—To provide further evidence that HSP90-α is a SOXF, human recombinant HSP90-α (hrHSP90-α) was studied. The preparation of hrHSP90-α used for these studies was highly purified as shown by silver stain analysis, which revealed that >95% of total protein migrated at a molecular mass of 90 kDa, consistent with hrHSP90-α protein (Fig. 12A). hrHSP90-α stimulated ERK1/2 activity in VSMC in a concentration-dependent manner (Fig. 12B and C). The time course for ERK1/2 activation of growth-arrested VSMC by 10 nM hrHSP90-α (Fig. 13) was similar to that observed with conditioned medium with peak at 10 min (Fig. 13B).

**DISCUSSION**

The major finding of the present study is that oxidative stress induced by the naphthoquinolinedione LY83583 stimulates VSMC to release protein factors that activate ERK1/2. By use of sequential chromatography and tandem mass spectrometry we identified several proteins specifically secreted in response to oxidative stress that we term SOXF for secreted oxidative stress-induced factors. These proteins include HSP90-α and cyclophilin B as well as several unidentified proteins. Generation of SOXF was a common feature of agonists that produced O$_2$ in VSMC including LY83583, menadione, and xanthine plus xanthine oxidase. However, SOXF did not require generation of O$_2$ to activate ERK1/2 as shown by the failure of Tiron to inhibit SOXF-stimulated ERK1/2 activity. It is important to note that SOXF stimulation of ERK1/2 may be the consequence of several different proteins acting alone or in concert as multiple proteins were released in response to oxidative stress and co-purified with SOXF activity.

Secretion of intracellular proteins in response to heat shock and inflammation has been widely reported. In fact, several members of the cyclophilin family, including cyclophilin A, cyclophilin B, and cyclophilin C, have been reported to be secreted and present in biological fluids including milk, plasma, and synovial fluid (20, 21). Release of these cyclophilins was specific and not due to cell lysis (21, 22). Release of SOXF by LY83583 from VSMC also appears to be specific and not a consequence of cell destruction as shown by lack of change in cell morphology, cell viability, or lactate dehydrogenase. Finally, several other cytoplasmic proteins, such as HSP90-β,
PKC-α, PKC-ζ, and c-Raf-1, were not present in the conditioned medium from LY83583-stimulated VSMC. However, the mechanism by which intracellular proteins that lack signal peptide sequences, such as HSP90-α, is secreted in response to oxidative stress remains unknown.

Heat shock proteins are a family of cellular proteins characterized by their up-regulation in response to stress, the presence of a weak ATPase activity, and a high degree of sequence homology. Heat shock proteins were initially described as chaperones that facilitate the folding of other proteins. However, recent studies now indicate that heat shock proteins also play a role in cytoprotection, anti-apoptosis, and signal transduction. For example, constitutive expression of HSP27 prevents Fas-mediated apoptosis (23), and induction of HSP70 prevents activation of c-Jun N-terminal kinase and p38 (24). Multiple species of heat shock proteins have been characterized in VSMC including HSP10, HSP27, HSP40, HSP60, HSP70, HSP75, HSP78, HSP90, and HSP110. Aortae from hypertensive rats express increased mRNA and protein for HSP70 (25) as compared with normotensive controls. Heat shock proteins may play a role in ischemic preconditioning because hearts from transgenic mice overexpressing HSP70 in the myocardium demonstrated enhanced functional recovery following brief episodes of ischemia sufficient to induce sustained abnormalities in nontransgenic littermates (26). It is important to note that the functions of extracellular HSP90-α described here may differ significantly from the currently described intracellular functions of heat shock proteins.

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**Fig. 12.** hrHSP90-α activates ERK1/2 in a dose-dependent manner in VSMC. A, hrHSP90-α (μg) protein purity was analyzed by SDS-PAGE followed by silver stain. B, growth-arrested VSMC were treated with the indicated concentrations of hrHSP90-α for 10 min. Cell lysates were prepared and analyzed for ERK1/2 activity and total ERK1/2 protein levels by Western blot. A representative Western blot of ERK1/2 activity (upper panel) and ERK1/2 protein levels is shown (lower panel). C, the results were quantified by densitometry of autoradiograms. Results were normalized to control (time = 0), which was arbitrarily set to 1.0. Results are mean ± S. D. of four experiments.

**Fig. 13.** Time course of ERK1/2 activation by hrHSP90-α. Growth-arrested VSMC were treated with 10 nM hrHSP90-α for the indicated times. Cell lysates were prepared and analyzed for ERK 1/2 activity and total ERK1/2 proteins by Western blot. A, a representative Western blot showing ERK1/2 activity (upper panel) and ERK1/2 protein levels (lower panel). B, the results were quantified by densitometry of autoradiograms. Results were normalized to control (time = 0), which was arbitrarily set to 1.0. Results are mean ± S. D. of three experiments.

Cyclophilins are a family of ubiquitous proteins that are abundant in nearly every cell type and highly conserved throughout evolution from man to bacteria, indicating that they likely serve a fundamental cellular function (27). Cyclophilins have been proposed to assist in protein folding, to act as chaperones, and to be chemotactic (27). Recent findings suggest potentially important roles for secreted cyclophilins in regulating cell growth (20) and inflammatory responses (21, 22). Although we unambiguously identified cyclophilin B in conditioned medium by tandem mass spectroscopy it appears that other cyclophilins including cyclophilin A may be secreted in response to oxidative stress.

The biological consequences of SOXF secretion have not been investigated in the present study. As a speculation we propose that SOXF secretion serves an autocrine and/or paracrine function during oxidative stress to promote cell survival and/or initiate tissue repair processes. The fact that HSP90-α, a chaperone that preserves protein conformation and is induced by many cell stresses, was among the proteins identified as a putative SOXF supports this concept. In summary, we have shown that several proteins are secreted specifically in response to oxidative stress in VSMC. Future studies will be required to investigate the functional role of SOXF candidate proteins under physiological conditions of oxidative stress such as ischemia/reperfusion.

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