Cytokine stimulation of pregnancy-associated plasma protein A expression in human coronary artery smooth muscle cells: inhibition by resveratrol

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Cytokine stimulation of pregnancy-associated plasma protein A expression in human coronary artery smooth muscle cells: inhibition by resveratrol. Am J Physiol Cell Physiol 290: C183–C188, 2006; doi:10.1152/ajpcell.00199.2005.—Through specific cleavage of proteins that bind and inhibit insulin-like growth factor-I (IGF-I), pregnancy-associated plasma protein-A (PAPP-A) enhances local IGF-I availability, and, consequently, receptor activation. PAPP-A expression is increased in experimental models of vascular injury and in human atherosclerotic plaque; however, little is known about the regulation of PAPP-A gene expression in vascular cells. In this study, we tested the hypothesis that proinflammatory cytokines involved in the vascular injury response stimulate PAPP-A gene expression in human coronary artery smooth muscle cells (hCASMC) in culture. Tumor necrosis factor (TNF)-α and interleukin (IL)-1β stimulated PAPP-A gene expression in a time- and dose-dependent manner. The effect of these cytokines appears to be at the level of transcription because actinomycin D completely prevented the induction of PAPP-A gene expression. Accumulation of PAPP-A in cell-conditioned medium paralleled mRNA synthesis, as did proteolytic activity against IGF binding protein-4 (IGFBP-4). Interestingly, pretreatment of hCASMC with resveratrol, a polyphenol found in the skin of grapes and in red wine protein (proMBP), and 28S were described previously (12, 37). Relative detection and amplification of PAPP-A, the precursor form of major basic protein (proMBP), and 28S were described previously (12, 37). Relative mRNA abundance was calculated using the 2-ΔΔCT method (30).

MATERIALS AND METHODS

Materials. Tumor necrosis factor (TNF)-α and interleukin (IL)-1β were obtained from Research Diagnostics (Flanders, NJ). IGF-I was provided by Dr. Martin Spencer (San Francisco, CA). Resveratrol and actinomycin D were obtained from Sigma-Aldrich (St. Louis, MO), and reagents for SDS-PAGE were from Bio-Rad Laboratories (Richmond, CA).

Cell culture and RNA preparation. hCASMCs were obtained from Clonetech (Walkersville, MD) and cultured in smooth muscle growth medium containing 5% fetal bovine serum (FBS) as described previously (6). Cells of the fourth to sixth passage were used for experiments. The cells were washed twice, preincubated overnight in medium containing 0.2% FBS, and then washed again and changed to 0.2% FBS with the indicated experimental additions. At the end of the incubation, conditioned medium was collected, centrifuged to remove debris, and stored at −70°C. Cell numbers were determined with the use of a Coulter Counter (Coulter Electronics, Hialeah, FL). Alternatively, total RNA was extracted from cells with the use of the RNaseasy Mini Kit (Qiagen, Valencia, CA) and treated with DNase (DNA-free; Ambion, Austin, TX). Four hundred nanograms of RNA were reverse transcribed with the use of TaqMan RT reagents (PE Biosystems, Foster City, CA) according to manufacturer’s instructions.

Real-time PCR. Real-time quantitative PCR analyses were performed using the ABI PRISM 7700 Sequence Detection System and software (PE Applied Biosystems). Primer and probe sequences for specific detection and amplification of PAPP-A, the precursor form of major basic protein (proMBP), and 28S were described previously (12, 37). Relative mRNA abundance was calculated using the 2-ΔΔCT method (30).
PAPP-A ELISA. PAPP-A levels in cell-conditioned medium were measured with the use of an Ultra-Sensitive PAPP-A ELISA kit (Diagnostic Systems Laboratories, Webster, TX). Minimum sensitivity was 0.24 mIU/l with intra- and interassay variation coefficients of 4.7% and 4.2%, respectively.

IGFBP-4 ELISA. IGFBP-4 levels in cell-conditioned medium were measured with the use of an IGFBP-4 ELISA kit from Diagnostic Systems Laboratories. Minimum sensitivity is 1 ng/ml, with intra- and interassay coefficients of variation of 2.8–6.4% and 2.3–6.7%, respectively. This assay recognizes both intact and proteolytically cleaved human IGFBP-4.

Protease assay. Cell-free IGFBP-4 proteolysis was assayed as previously described (6, 12, 13, 27, 37). Conditioned medium was incubated at 37°C for 2 h with 125I-IGFBP-4 and 5 nM IGF-IL. IGF-II binds IGFBP-4 and increases its susceptibility to cleavage by PAPP-A in vitro (35). Reaction products were separated by SDS-PAGE and visualized by autoradiography. Extent of proteolysis, i.e., loss of intact and generation of 18-kDa radiolabeled fragments, was determined using enhanced laser densitometry (LKB Ultroscan XL).

Statistical analyses. Data are presented as means ± SE. Statistical analyses were performed using ANOVA, followed by multiple comparisons. Results were considered statistically significant at P < 0.05, and were replicated in independent experiments.

RESULTS

Cytokine stimulation of PAPP-A expression. TNF-α and IL-1β were chosen to study the effects of proinflammatory cytokines on PAPP-A expression in cultured hCASMC. Dose-dependent increases in PAPP-A levels in hCASMC-conditioned medium were seen after treatment of cells with TNF-α and IL-1β for 24 h (Fig. 1). TNF-α showed half-maximal effectiveness at 1 pM, with maximal effectiveness (threefold) at 10 pM. IL-1β showed half-maximal effectiveness at ~0.01 pM, with maximal effectiveness (fourfold) at 1 pM. A time-course experiment of TNF-α- and IL-1β-stimulated PAPP-A mRNA expression in hCASMC indicated significant increases at 4 h and peak expression (15- to 25-fold elevated relative to control) at 8 h, with elevated expression (3 to 5 fold) retained at 24 h (Fig. 2). The effect of these proinflammatory cytokines appears to be at the level of transcription, as the RNA poly-

Fig. 1. Pregnancy-associated plasma protein-A (PAPP-A) protein levels: dose-dependent effects of TNF-α and IL-1β. Conditioned media (24 h) from human coronary artery smooth muscle cells (hCASMC) treated without and with the indicated concentrations of tumor necrosis factor-α (TNF-α; open symbols) or interleukin-1β (IL-1β; closed symbols) were assayed for PAPP-A by ELISA. Results are the means ± SE of triplicate determinations.

Fig. 2. PAPP-A mRNA levels: time course effects of TNF-α and IL-1β. Real-time RT-PCR was performed on RNA isolated from hCASMC after 1, 4, 8, and 24 h of treatment without (control, solid bars) and with 1 nM TNF-α (light gray bars) or IL-1β (dark gray bars). PAPP-A mRNA abundance is expressed relative to control at each time point. Results are means ± SE of triplicate determinations. *P < 0.05, significant difference vs. control.

Fig. 3. Effect of actinomycin D on TNF-α- and IL-1β-stimulated PAPP-A expression. hCASMC were treated for 6 h without (control, Ctl) or with 1 nM TNF-α or IL-1β in the absence or presence of actinomycin D (ActD; 0.5 μg/ml). Results are the means ± SE of triplicate determinations. *P < 0.05, significant difference vs. control; ‡P < 0.05, significant effect of ActD.

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merase inhibitor, actinomycin D, completely prevented TNF-α and IL-1β induction of PAPP-A gene expression (Fig. 3). Treatment of hCASMC with these cytokines had no significant effect on total (intact and fragmented) IGFBP-4 levels (Table 1).

Resveratrol inhibition of PAPP-A expression. Resveratrol, a polyphenol abundantly present in the skin of grapes and in red wine, is a promising cardiovascular system-protective agent (8, 39, 42, 44). We found that pretreatment of hCASMC with resveratrol at 30 μM, a dose determined to be maximally effective in initial experiments, markedly inhibited TNF-α- and IL-1β-stimulated PAPP-A mRNA synthesis (Fig. 4A) and protein expression (Fig. 4B). Resveratrol had no significant effect on basal PAPP-A expression. Resveratrol had no significant effect on cell number in these experiments (Table 2).

IGFBP-4 proteolysis. To substantiate that the changes in PAPP-A expression regulated by cytokines and resveratrol correlate with changes in proteolytic activity, we assessed proteolytic activity against 125I-labeled IGFBP-4 in cell-conditioned medium (Fig. 5). In a cell-free assay, conditioned medium from hCASMC exhibited basal IGFBP-4 protease activity (lane b), as previously reported (6). However, medium from cells treated with TNF-α (lane d) and IL-1β (lane f) showed enhancement of proteolytic activity against IGFBP-4. In three separate experiments, cytokine-stimulated protease activity was two- to threefold that of basal activity. Importantly, the cytokine-stimulated increase in activity was inhibited by resveratrol treatment of the cells (lanes e and g) without any effect on the basal level of IGFBP-4 proteolytic activity (lane c).

DISCUSSION

The data presented here demonstrate that PAPP-A gene expression in hCASMC is stimulated by proinflammatory cytokines and suggest a mechanism for the regulation of PAPP-A in response to vascular injury that may contribute to the enhanced IGF-I bioactivity in intimal hyperplasia and atherosclerotic plaque development. Furthermore, resveratrol, currently under investigation for its potential antioxidant and...
cardioprotective properties (8, 39, 42, 44), was able to block cytokine-stimulated PAPP-A expression.

It is well established that IGFs and cytokines are critically involved in the vascular response to acute and chronic injury (5, 14, 24, 38), but there has been little investigation into a possible relationship between these two systems. The proinflammatory cytokines TNF-α and IL-1β have both been implicated in the regulation of atherosclerotic plaque development and stability. Thus, our finding that TNF-α and IL-1β stimulate hCASMC synthesis of PAPP-A is an important linkage because PAPP-A cleaves the inhibitory IGFBP-4, resulting in increased local IGF-I bioavailability and receptor signaling (10, 13, 34). We observed a log difference in dose effectiveness between the two cytokines (IL-1β > TNF-α), but they showed very similar stimulation kinetics with an increase in PAPP-A mRNA expression at 4 h that peaked ~8 h and continued to be elevated relative to controls 24 h poststimulation. The increases in PAPP-A levels in the cell-conditioned medium reflected the large increase in PAPP-A mRNA synthesis. Because the stimulation of PAPP-A expression was blocked by actinomycin D, the effects of TNF-α and IL-1β appear to be at level of transcription.

Receptors recognizing and responding to TNF-α and IL-1β are present on vascular SMC (23, 24). This is the first report to demonstrate marked increases in SMC expression of PAPP-A, a metalloprotease in the metzincin superfamily (7), on treatment with these cytokines. Several members of the matrix metalloproteinase family, which also belong to the metzincin superfamily, have been implicated in plaque development and instability through their functional role in degrading extracellular matrix components (15, 17, 29, 31, 36, 41). PAPP-A has not been associated with matrix degradation in vivo or in vitro, but rather exclusively with specific cleavage of IGFBPs (25). However, we cannot exclude the possibility that PAPP-A has other substrates.

Resveratrol is naturally found at high concentrations in grapes and derived products, such as red wine, and it has been proposed that this compound is the cardioprotective agent behind the “French paradox” (8, 39, 42, 44). Resveratrol possesses many biological activities that favor protection against atherosclerosis, including inhibition of vascular SMC proliferation (2, 22, 33). In this study, we show that resveratrol blocks cytokine-stimulated PAPP-A expression in hCASMC without affecting basal expression. The decrease in measured protease activity on treatment of cells with resveratrol paralleled the decrease in PAPP-A mRNA and protein levels, suggesting a direct effect of resveratrol on PAPP-A expression. The observed decrease in proteolytic activity was not due to an induction of proMBP (data not shown), a physiological inhibitor of PAPP-A, which in other studies has been shown to account for a decrease in IGFBP-4 protease activity on treatment of human fibroblasts with phorbol ester tumor promoters (12). Several studies investigating the cellular mechanism of resveratrol have shown a reduction in the activation of NF-κB (21, 43), a transcription factor that is involved in mediating oxidative stress, which is upregulated in atherosclerosis (9, 11, 28). TNF-α-induced human vascular SMC proliferation is mediated by NF-κB (40). TNF-α and IL-1β also activated NF-κB in hCASMC, but this activation was not affected by resveratrol (data not shown). Further studies will be necessary to determine the mechanism by which resveratrol inhibits cytokine-induced PAPP-A expression.

The data presented here are consistent with a proposed model in which an increase in PAPP-A expression stimulated by proinflammatory cytokines, such as TNF-α and IL-1β, amplifies the vascular response to IGFs through PAPP-A-induced cleavage of IGFBP-4 (Fig. 6). The most abundant IGFBP in the vascular wall is IGFBP-4, which is secreted by vascular SMC. Intact IGFBP-4 binds IGF-I, forming a pericellular reservoir of this potent growth factor. Vascular SMCs have receptors for IGF-I and respond to IGF-I with increased signaling leading to proliferation, migration, and extracellular matrix production. However, under the conditions depicted in

Fig. 5. Proteolytic activity against IGFBP-4: regulation by TNF-α, IL-1β, and resveratrol. Lane a shows unconditioned medium. hCASMC-conditioned media from control cells (lane b) or cells treated with resveratrol (lane c), TNF-α (lane d), TNF-α + resveratrol (lane e), IL-1β (lane f), IL-1β + resveratrol (lane g) were incubated with [125I]insulin-like growth factor binding protein-4 (IGFBP-4), as described in MATERIALS AND METHODS. Reaction products were separated by SDS-PAGE, and the gel was dried and exposed to film. Arrows indicate intact and cleaved IGFBP-4.

Fig. 6. Proposed model for PAPP-A regulation of the vascular response to IGF-I. A: quiescent state of vascular smooth muscle cells (SMCs). IGF-I is inactivated by its tight binding to IGFBP-4, which is synthesized by the vascular SMC. B: cytokine-stimulated PAPP-A expression and consequent IGF response. PAPP-A, secreted by vascular SMC, binds to the cell surface through domains in the COOH-terminal end causing proteolytic release of IGF-I to occur in proximity to the IGF receptor. See text for further details.
Fig. 6A, vascular SMCs are relatively quiescent. Vascular SMC also have receptors for proinflammatory cytokines, which are available in vascular tissue, e.g., when secreted by activated macrophages. These molecules, released in the injured area, stimulate PAPP-A expression by SMCs causing cleavage of IGFBP-4 at the surface (26) and, consequently, increased local IGF. IGF-1 receptor activation during response to injury results in increased proliferation, migration, and matrix production (Fig. 6B). This model could apply to acute injury to the vasculature, such as in restenosis after balloon angioplasty and stenting, as well as to chronic injury, such as in atherosclerosis. In addition, possible cytokine-modulated changes in IGF-I, IGF-1 receptor, and/or IGFBP expression in vascular SMC (1, 45) may be relevant to this model.

Our results further suggest that the cardioprotective effects of resveratrol may, in part, be caused by its blocking of cytokine-stimulated PAPP-A expression, although such a model awaits direct testing in vivo. A further increase in our understanding of the mechanism of cytokine regulation of IGF bioavailability in general and PAPP-A expression in particular may lead to novel therapeutic targets for intimal hyperplasia and atherosclerosis.

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