Oxytocin attenuates NADPH-dependent superoxide activity and IL-6 secretion in macrophages and vascular cells

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Cell cultures. Endothelial cells were cultured in Medium 200 supplemented with low-serum growth supplement (Invitrogen). Smooth muscle cells were cultured in basal medium (Lonza) containing supplements and growth factors at 37°C in a humidified 95% air-5% CO2 incubator. Cells were used between the third and eighth passage. For experiments, cells were plated into 35- or 60-mm dishes at 25,000 or 50,000 cells/dish and grown until ~80% confluent.

THP-1 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.05 μM mercaptoethanol. Cells were seeded at 100,000 cells/well in 24-well tissue culture plates or into 60- or 100-mm dishes at 1 × 10^5 or 5 × 10^5 cells/dish, respectively, and differentiated by adding 100 ng/ml PMA for 24–48 h and maintained for 7–8 days before experimental manipulation.

Cells were incubated with oxytocin at physiologically relevant levels (10–100 pM) on the basis of studies reporting plasma oxytocin concentrations of 5–250 pM (1, 20, 27).

Oxytocin receptor protein expression. Oxytocin receptor expression was examined in cell membrane fractions. Phosphate-buffered saline (PBS)-washed cells were collected by scraping and suspended in lysis buffer (15 mM KCl, 1.5 mM MgCl2, 10 mM HEPES, 1 mM dithiothreitol, 250 mM sucrose, pH 7.4). Cells were homogenized using a dounce homogenizer then centrifuged for 5 min at 800 g. The resulting supernatant was centrifuged for 10 min at 10,000 g at 4°C to remove subcellular fractions. The supernatant was then centrifuged for 60 min at 100,000 g at 4°C to obtain membrane-enriched pellets. The pellet was solubilized in SDS buffer (50 mM Tris, pH 6.8, 2% SDS), aided by brief sonication. Protein was measured with BCA Protein Assay (Pierce, Rockford, IL).

For immunoblotting, 10–20 μg of protein was separated by denaturing and reducing electrophoresis on 10–20% Tris-glycine polyacrylamide gradient gels (Lonza). After separation, proteins were transferred to nitrocellulose. Membranes were blocked in Tris-buffered saline containing 5% nonfat powder milk and 0.1% Tween-20 for 1 h and then probed with polyclonal anti-goat oxytocin receptor (ADI, San Antonio, TX) diluted in blocking buffer (1:200) at 4°C overnight. Immunoreactive bands were detected with appropriate peroxidase-conjugated secondary antibody for 1 h and then visualized with chemiluminescence. To normalize for protein loading, the membranes were stripped and reprobed with anti-actin antibody.

Deglycosylation of oxytocin receptor. THP-1 macrophages were collected, and membrane pellets were prepared as described above. Peptide N-glycosidase (PNGase-F; Sigma) was used for the deglycosylation of the mature oxytocin receptor overnight at 37°C. The enzyme reaction was terminated by addition of SDS buffer. The samples were subjected to electrophoresis and immunoblotted with oxytocin receptor antibody.

PCR experiments for oxytocin receptor. Oxytocin receptor mRNA expression was evaluated by polymerase chain reaction (PCR). Total RNA (optical density ratio of 260/280 nm, >1.8) was isolated using the RNeasy kit (Qiagen, Valencia, CA) and stored at −80°C. After treatment with DNase I (Applied Biosystems), the reverse transcriptase reaction was carried out by mixing 5–10 μg of total RNA in the presence of random oligomers (3.2 μg), MgCl2 (5 mM), deoxynucleotide mix (1 mM), AMV reverse transcriptase (10 U), and RNase inhibitor (50 U) and incubated at 25°C for 20 min, then at 42°C for 60 min, and then denatured at 99°C for 5 min and then cooled to 4°C for 5 min, resulting in cDNA synthesis.

Quantitative gene expression of human oxytocin receptor with the use of real-time PCR was performed with the TaqMan gene expression assay. Fifty nanograms of cDNA was amplified with TaqMan Universal PCR Master Mix and reactions run using universal cycling conditions on an Applied Biosystems 7500 Real-Time PCR system. Samples were analyzed in triplicate. The ΔΔCt (threshold cycle) method was used to analyze changes in gene expression in a given sample relative to another reference sample and expressed as the fold change in gene expression. 18S RNA was used as endogenous control. A no-template control was performed to ensure that there was no amplification of genomic DNA.

Effect of oxytocin on ERK1/2 activity. Phosphorylated ERK1/2 protein relative to total ERK1/2 protein was measured in all cell lines using Bioplex phosphoprotein and total target assays (Bio-Rad, Hercules, CA). For these studies, all cell lines were serum starved for 24 h in medium containing 1 mg/ml bovine serum albumin and then incubated for 10 min with 100 pM oxytocin. Cells were chilled on ice, washed three times with ice-cold PBS, and collected by scraping on ice with 250 μl of lysate buffer. Lysates were clarified by centrifugation at 20,000 g at 4°C and stored at −20°C until assayed.

NADPH oxidase activity. NADPH-dependent superoxide activity was measured by lucigenin-enhanced chemiluminescence (23). Briefly, cells in culture dishes were detached using 0.25% trypsin-EDTA, washed with PBS, and resuspended at 10^6 cells/ml in PBS-diethylenetriamine-pentaacetic acid (2 mM), and 2.5 × 10^5 cells/well were used for assay in a 96-well black microtiter plate. For cell homogenates, cultured cells were collected by scraping into PBS and lysed by brief sonication (2 × 5-s pulses using a cup horn sonicator probe at ~50% power). Protein was determined by the BCA Assay.

The reaction was initiated by addition of NADPH (100 μM) and dark-adapted lucigenin (5 μM). Light emission was recorded for 8 min with a luminometer (Centro LB 960; Berthold). Data were expressed as relative light units (RLU) normalized to protein content or cell number and corrected for a sample blank. Each experiment was performed in triplicate.

Control studies using vascular cells and macrophages demonstrated that cellular superoxide generation required NADPH and was nearly abolished by tiron (5 mM), a nonenzymatic scavenger of superoxide. The NADPH oxidase inhibitor diphenyleneiodonium (10 μM) reduced activity by >80%, whereas the respiratory mitochondrial chain inhibitor rotenone (50 μM) reduced superoxide production by <20%, indicating that the bulk of the superoxide formed was mediated by NADPH oxidase activity (21, 23).

IL-6 assay. Cells were incubated with LPS (100 ng/ml) or LPS with oxytocin (10 or 100 pM) for 6 h, and then cell-free supernatants were assayed for IL-6 using commercially available reagents (BD Biosciences, San Diego, CA).

Statistical analyses. Data were presented as means ± SE obtained in at least three separate experiments. Results were compared by paired t-tests or ANOVA with post hoc Tukey tests between experimental and control treatments. All analyses were performed using SPSS for Windows (Chicago, IL). An α-level of 0.05 was required for statistical significance.

For NADPH oxidase activity, a repeated-measures ANOVA was performed with time (min) as the repeated factor and oxytocin dose (control vs. oxytocin dose) as the between-groups factor. The dependent variable was the mean RLU across time (min). Student’s paired t-test was used to compare total RLU between experimental and control treatments.

RESULTS

Oxytocin receptor expression. Expression of oxytocin receptor in endothelial and smooth muscle cells, monocytes, and macrophages was examined. Although endothelial and smooth muscle cells normally exist in the vessel wall, infiltration of monocytes and macrophages occurs in response to inflammatory stimuli, as in atherosclerotic disease. Oxytocin receptor mRNA levels were quantified using real-time PCR (Fig. 1), and results were normalized to expression of 18S. Endothelial and smooth muscle cells express oxytocin receptor mRNA, confirming previous results (33, 37). Smooth muscle cells exhibited 3,600-fold higher mRNA levels relative to endothelial cells. THP-1 monocytes also expressed oxytocin receptor.
gene. Upon differentiation to macrophages, there was a further 10-fold increase in message levels relative to monocytes. Oxytocin receptor protein expression was also examined in these cells by immunoblotting (Fig. 2A). There was an immunoreactive band at 67 kDa molecular weight (MW) for endothelial cells, smooth muscle cells, monocytes, and macrophages consistent with the expected MW of the mature glycosylated form of the receptor. There was a fourfold increase of protein expression in THP-1 monocytes and macrophages compared with endothelial cells when normalized for total cell protein; however, there was no difference in expression of the 67-kDa band between the THP-1 monocytes or macrophages. In addition, there was a lower MW immunoreactive band in THP-1 monocytes and macrophages consistent with the molecular size expected for the unglycosylated form of the receptor (~46 kDa) (17). Upon differentiation of monocytes into macrophages, there was a 15-fold increase in expression of the 46-kDa band.

Cellular localization for oxytocin receptor was examined after subcellular fractionation of THP-1 macrophages (Fig. 2B). Protein expression of oxytocin receptors was also documented in two preparations of isolated human peripheral blood mononuclear cells and in the mouse macrophage cell line RAW 264.7 (data not shown). Upon differential centrifugation, there was a 67-kDa immunoreactive band in the plasma membrane-enriched fraction (100,000 g × 60 min) that was not present in the 10,000-g pellet. The 10,000-g fraction showed an immunoreactive band at the 46-kDa MW that was not apparent in the plasma membrane-enriched fraction. These data indicate that the mature form of the oxytocin receptor is enriched in the plasma membrane fraction. Taken together, our results indicate that oxytocin receptors are expressed in monocytic and macrophage cell lines.

To further establish that the 46-kDa band represented the unglycosylated form of the oxytocin receptor, the membrane pellets that contained predominantly the 67-kDa MW band of THP-1 macrophages were treated with PNGase-F. There was a shift in MW from 67 to 46 kDa after deglycosylation (Fig. 2C), supporting the notion that the 46-kDa band represents the unglycosylated form of the receptor and that glycosylation may be required for plasma membrane localization.

**Effect of oxytocin on ERK1/2 phosphorylation.** Oxytocin regulates a variety of physiological functions through its G protein-coupled receptor. Oxytocin receptor activation stimulates the ERK1/2 MAPK pathway in myometrial cells (7, 38) and in oxytocin receptor-transfected Chinese hamster ovary cells (32). To examine oxytocin receptor function, the amount of phosphorylated ERK1/2 protein relative to total ERK1/2 protein was quantified in all cell lines after treatment with oxytocin. Endothelial and smooth muscle cells, THP-1 monocytes, and macrophages treated with 100 pM oxytocin resulted in a significant increase in ERK1/2 phosphorylation compared with control cells (P < 0.05; Table 1). These data suggest that oxytocin is able to activate its receptor in all of the cell types examined.

**NADPH oxidase activity.** Oxytocin has been suggested to reduce oxidant stress (12, 13, 28). To explore this possibility, the effect of oxytocin on cellular superoxide production mediated by NADPH oxidase activity was measured. When THP-1 monocytes were incubated with 10 pM oxytocin, there was a 25% decrease in total NADPH superoxide production (P < 0.05).
0.001 relative to control; Fig. 3A). NADPH oxidase activity was also measured in THP-1 macrophages differentiated with PMA (Fig. 3B). Differentiation of the THP-1 cells increased NADPH-dependent superoxide production fourfold compared with monocytes (compare values in Fig. 3, A and B). Incubation with 10 pM oxytocin decreased total NADPH superoxide production in macrophages by 48% ($P < 0.001$ relative to control). No further decrease in NADPH-dependent superoxide production was observed at 100 or 1,000 pM oxytocin (data not shown).

The effects of oxytocin on NADPH oxidase activity in endothelial cells (Fig. 3C) and smooth muscle cells (Fig. 3D) were similarly studied. Incubation with 10 pM oxytocin decreased NADPH-dependent superoxide production by 24% in both aortic endothelial cells ($P < 0.01$) and smooth muscle cells ($P < 0.05$) compared with controls.

NADPH-dependent superoxide production increased in TNF-stimulated endothelial cells by 35% ($P < 0.05$) compared with control (Fig. 4). When endothelial cells were preincubated with 100 pM oxytocin for 1 h and then stimulated with TNFα for 30 min, there was a 36% decrease in NADPH oxidase activity compared with TNF-stimulated endothelial cells ($P < 0.05$). These data show that oxytocin attenuated TNF-mediated activation of NADPH-dependent superoxide in endothelial cells.

**Effect of oxytocin on IL-6 secretion in LPS-stimulated THP-1 macrophages and endothelial cells.** The effect of oxytocin on IL-6 secretion was examined in LPS-stimulated THP-1 macrophages and aortic endothelial cells. THP-1 macrophages and endothelial cells not stimulated with LPS, including those treated with oxytocin alone, showed minimal to no IL-6 secretion (<4 pg/ml; data not shown). Incubation with LPS (100 ng/ml) stimulated IL-6 secretion, and addition of oxytocin resulted in a dose-dependent attenuation of LPS-stimulated IL-6 secretion. There was a 36% decrease in IL-6 secretion when LPS-stimulated macrophages were treated with 10 pM oxytocin ($P < 0.01$) and a 56% inhibition in cells treated with 100 pM oxytocin ($P < 0.001$ vs. LPS-stimulated macrophages) (Fig. 5A).

LPS-stimulated endothelial cells were also treated with 100 pM oxytocin, which resulted in a 26% decrease in IL-6 secretion ($P < 0.001$ vs. LPS-stimulated cells; Fig. 5B). Similar experiments were done in aortic smooth muscle cells; however, these cells exhibit constitutively high levels of IL-6 secretion.
that was not affected by oxytocin treatment (not shown). Taken together, these data demonstrate that oxytocin attenuates IL-6 secretion from LPS-stimulated THP-1 macrophages and endothelial cells.

**DISCUSSION**

The current study demonstrated in an in vitro model that oxytocin has antioxidant and anti-inflammatory effects on vascular cells and THP-1 macrophages. Oxytocin receptors were identified in all cell types examined, and oxytocin decreased NADPH-dependent superoxide production and IL-6 secretion in these cells. By reducing oxidative stress and inflammatory processes in cells present in the vessel wall, these data suggest that oxytocin may have antiatherogenic properties.

**Oxytocin receptors on vascular cells, monocytes, and macrophages.** Oxytocin receptor protein and mRNA were identified in aortic endothelial cells and smooth muscle cells, confirming previous data (33, 37). A novel finding was the demonstration of this protein and mRNA in human THP-1 monocytes and macrophages and in two preparations of isolated human peripheral blood monocytes. Oxytocin receptor mRNA was present in aortic endothelial and smooth muscle cells, THP-1 monocytes and macrophages; however, the relative level of oxytocin transcript varied. Using quantitative real-time PCR, there was an approximately 3,600-fold increase in oxytocin receptor mRNA levels in aortic smooth muscle cells compared with endothelial cells. The finding of large differences in mRNA levels between cell types was unexpected considering comparable protein levels among cell types. The discordance between mRNA and protein expression may be due to differential regulation of the receptor at the message or protein level. The relationship between mRNA and protein levels is not well understood and suggests complex regulation of the receptor.

By Western blotting, all cell types examined expressed an immunoreactive band consistent with the expected MW of the mature form of the receptor (18). Additionally, macrophages exhibited a lower (46 kDa) immunoreactive band in THP-1 monocytes and macrophages that was increased 10-fold upon differentiation to macrophages. Studies in uterine tissue have also identified different MW forms of the oxytocin receptor (19). PNGase-F studies support the concept that the lower MW band represents the unglycosylated form of oxytocin receptor. One possibility may be that different glycosylation sites on the receptor may play a role in ligand binding and affinity; however, in vitro studies found that disruption of these glycosylation sites did not affect binding function (17). However, in order for receptors to be functional for targeting their ligands, receptors must be expressed on the cell surface. Differential centrifugation suggests localization of the receptor in different fractions. Further studies are needed to confirm cellular localization and functionality of the different receptor forms.

**Oxytocin, NADPH oxidase activity, and IL-6 release.** Oxidative stress and inflammation play an important role in ath-

![Fig. 4. Effect of oxytocin on total NADPH-dependent superoxide production in TNF-stimulated endothelial cells. Data are means ± SE and from 3 independent experiments. *P < 0.05 vs. TNF-stimulated cells.](http://ajpendo.physiology.org/)

![Fig. 5. Effect of oxytocin on IL-6 secretion from LPS-stimulated cells. THP-1 macrophages (A) and endothelial cells (B) treated with LPS (100 ng/ml) were incubated with 10 or 100 pM oxytocin, and IL-6 secretion was measured after 6 h. Data are means ± SE and from 3 independent experiments. *P < 0.01, **P < 0.001 vs. LPS-stimulated cells.](http://ajpendo.physiology.org/)
erosclerosis (3, 22, 29). The current study examined the effects of oxytocin on specific aspects of these disease processes. NADPH oxidase is the major source of superoxide production in the vascular wall (2, 9). A novel finding in the current study was the oxytocin-induced reduction in NADPH oxidase activity in aortic endothelial and smooth muscle cells, THP-1 monocytes, and macrophages. Additionally, oxytocin diminished the induction of NADPH superoxide production by TNFα. A possible explanation for the decreased NADPH oxidase activity was that oxytocin was acting as a superoxide scavenger. It was shown that oxytocin scavenged free radicals and prevented lipid peroxidation at supraphysiological concentrations of oxytocin (0.2–20 μM) (25). Control studies using xanthine-xanthine oxidase to generate superoxide in the presence of up to 1 μM oxytocin had no effect on the measurement of superoxide production, suggesting that oxytocin was not acting as a nonspecific scavenger of superoxide (data not shown).

To assess the putative anti-inflammatory effects of oxytocin, the current study examined IL-6 secretion in oxytocin-treated THP-1 macrophages and endothelial cells. Oxytocin decreased LPS-stimulated IL-6 secretion from macrophages by 56% and endothelial cells by 26%, suggesting an attenuation of inflammatory processes in these cells. This finding is interesting in light of the fact that oxytocin was shown to decrease cytokines in response to LPS in men (5) and that the oxytocin receptor gene contains an IL-6 response element that upregulates the receptor in response to inflammation (16, 30, 31). Because macrophages are found throughout the body, these findings also suggest that oxytocin may have a broader anti-inflammatory function in tissues other than the vasculature.

Social environment, atherosclerosis, and oxytocin. Previous studies from our laboratory demonstrated that a stable social environment, characterized by increased affiliative social behavior, slowed the progression of atherosclerosis in an animal model (24, 27). In addition, a separate study suggested that social environment differentially modulated inflammatory and oxidative stress mechanisms associated with disease progression (26). Given the extensive literature that relates social behavior to oxytocin and the findings of the current study, it is proposed that elevations in peripheral or local oxytocin as a function of social environment could work directly on vascular cells and macrophages to slow the progression of atherosclerosis. Future studies will examine the mechanisms linking social behavior, oxytocin, and disease.

In summary, oxytocin receptors were identified in cultured aortic endothelial and smooth muscle cells, as well as THP-1 monocytes and macrophages, and the effects of oxytocin on oxidative stress were examined to understand the role of oxytocin in the atherogenic process. The presence of oxytocin receptors were identified on THP-1 monocytes and macrophages for the first time and confirmed in aortic endothelial and aortic smooth muscle cells. NADPH oxidase activity and IL-6 secretion were decreased in vascular cells treated with oxytocin. These novel findings suggest that oxytocin may contribute to an additive effect of decreased superoxide production and decreased IL-6 release that may slow the progression of atherosclerosis.

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