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Substance P Stimulates Vascular Endothelial Cellular Reducing Capacity in the Presence of Insulin and Human Plasma Factors

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Abstract Substance P (SP) is an important tachykinin in vascular wall biology. In previous studies [Villablanca et al. (1994): Circ Res 75:1113–1120], the authors have demonstrated that SP is a stimulus for endothelial cell growth and proliferation in serum-free culture conditions with cells quiescent in the G_0–G_1 phase of the cell cycle. As mitogenic and metabolic activity may interrelate, the purpose of this study was to determine the effects of the vasoactive perivascular neuropeptide SP on changes in the metabolic function of endothelial cells, and to characterize the response, by studying cellular reducing capacity in aortic vascular endothelial cells. In addition, interactions between SP and other growth factors (insulin and non-platelet plasma factors) were investigated and compared to the responses to SP alone. Metabolic effects were determined by evaluating cellular reducing capacity by the conversion of (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) to formazan (the MTT assay). The findings demonstrated that SP alone (10 pg/ml-25 µg/ml) inhibited cellular reducing capacity in vascular endothelial cells. In contrast, SP in the presence of insulin (10 µg/ml) stimulated endothelial reducing capacity, as compared to SP alone, by twofold on average. The effect of SP and insulin was additive at ⩽0.001 µg/ml SP, and synergistic at SP concentrations ranging within 0.01–1.0 µg/ml. SP in the presence of human platelet-poor plasma (HPPP, 5%) stimulated endothelial reducing capacity, as compared to SP alone, by threefold on average. The effect of SP and HPPP was additive at ⩽0.01 µg/ml SP and synergistic at SP concentrations of 0.1–25 µg/ml. Lastly, SP in the presence of insulin and HPPP stimulated endothelial metabolic activity, as compared to SP alone, by 14-fold on average. An additive response to SP, insulin, and HPPP was observed at the lowest SP concentration studied (10 pg/ml). At all other SP concentrations studied (0.0001–25 µg/ml), the responses to insulin, HPPP, and SP were synergistic. Our studies indicate that the vasoactive neuropeptide substance P may synergize with insulin and HPPP in regulating endothelial cell metabolism. In addition, our findings suggest that the mechanisms by which SP stimulates cellular metabolism are different from the mechanisms by which it stimulates cell growth. J. Cell. Biochem. 66:471–481, 1997.

Key words: substance P; cell cycle; cell growth; endothelial cell; tachykinin; nitric oxide; insulin; plasma; MTT

The endothelium consists of a quiescent, contact-inhibited, functionally complex cell monolayer. It plays a role in a variety of cellular events including the elaboration of growth factors and regulation of vascular cell growth, and is important pathophysiologically in atherogenesis and wound healing [Ross, 1986].

Substance P (SP) is a ubiquitous vasoactive perivascular member of the tachykinin family of neuropeptides, which share a common C-terminal sequence. It is present in primary afferent nerve fibers enveloping the vasculature with a high density of SP-containing networks present in the aorta and vena cavae close to the heart [Furness et al., 1982]. SP has also been localized in the adventitia and the medial–adventitial border of the vessel wall and is synthesized and released from arterial endothelial cells [Linnick and Moskowitz, 1989; Loesch and Burnstock, 1988; Ralevic et al., 1990]. SP is present in the blood of humans and other mammals and can be detected in plasma by radioim-

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munoassay (RIA) [Skrabanek et al., 1976] with increased plasma levels reported in conditions characterized by uncontrolled cellular growth including malignancies [Powell et al., 1980].

A variety of circulatory and vascular effects of SP have been elucidated or postulated including its role in tissue repair [Shimizu et al., 1984], increased vascular permeability [Nguyen et al., 1995], inflammation [Matis et al., 1990], and peripheral and coronary vasodilation and modulation of coronary blood flow [Christie et al., 1989; Nakamura et al., 1990]. The two latter effects are associated with SP-mediated endothelial prostacyclin (PGI_2) [Marceau et al., 1989] and endothelium-derived nitric oxide (NO) production [Bossaller et al., 1989].

Evidence from previous studies [Nilsson et al., 1985; Payan, 1985] suggests that sensory neuropeptides may also play an important role in regulating in vitro cellular proliferation. We have demonstrated that SP is a stimulus for arterial endothelial cell (EC) growth in a serum-free culture environment when measured by cell counts and DNA synthesis [Villablanca et al., 1994]. In addition, the growth response showed synergism with insulin, calcitonin gene-related peptide, and human plasma factors. Furthermore, SP has been demonstrated to play a role in a variety of cellular metabolic functions. However, it is unknown whether the role of SP on endothelial cell growth is associated with changes in cellular reducing capacity and energy metabolism. As mitogenic and metabolic activity may relate, we wished to study the effect of SP on vascular cell metabolic activity. To determine whether SP stimulated changes in cellular reducing capacity in vascular endothelial cells, and to characterize the metabolic response, we studied cellular reducing capacity in response to SP, as well as the modulating effects of known growth factors and blood elements, including insulin and human platelet-poor plasma (HPPP). Cellular responses were studied by determining cellular metabolic activity as detected by the tetrazolium bromide salt assay (MTT assay).

METHODS

Materials

Chemicals. SP was obtained from Peninsula Laboratories (Belmont, CA). Aqueous solutions of SP were found to be unstable with repeated freeze-thawing but could be kept at 5°C for up to 1 week with loss of one-half of the maximal activity. All solutions were made up fresh prior to their use. SP was determined to be greater than 99% pure by high-performance liquid chromatography (HPLC) done in our laboratory on the purchased peptide. Bovine insulin and (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide), (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO), N,N-dimethylformamide (DMF), and sodium dodecyl sulfate (SDS) were purchased from Fisher Scientific (Pittsburgh, PA).

Tissue culture supplies. M199 was purchased from Fisher Scientific; fetal bovine serum (FBS) and iron-supplemented calf serum from Hyclone (Logan UT); penicillin/streptomycin/fungizone, trypsin, and ethylenediamine tetraacetic acid (EDTA) from Sigma; tissue culture plates from Falcon (Lincoln Park, NJ); tissue culture flasks from Corning Glass Works (Corning, NY); and cell scrapers from Costar (Van Nuys, CA).

Cell Culture

Secondary cultures of calf pulmonary artery (CPA) endothelial cells were obtained from the American Type Culture Collection (CRL 1733) (Rockville, MD) and as a generous gift from Una Ryan (St. Louis, MO). Cells were routinely cultured in M199 supplemented with 5% FBS, 5% iron-supplemented calf serum, and 10^{-5} M thymidine, hereafter referred to as growth media, plus antibiotics (penicillin 100 U/ml, streptomycin 0.1 mg/ml, and amphotericin B 0.25 U/ml) on plastic culture flasks in a humidified atmosphere of 5% CO_2 and air at 37°C. Cells were passaged weekly, nonenzymatically, at a split ratio of 1:2 to 1:4, using a cell scraper and were subcultured an average of 3–4 times (range 1–10 times) after they were received and before they were used for studies. No differences were seen in the cellular responses to neuropeptides and growth factors for the range of subcultures used.

MTT Assay

For this assay, endothelial cells were plated onto 96-well plates. In order to perform accurate counts for plating, cells were removed from the tissue culture flasks, by incubating them for 2 min at 37°C with 0.25% trypsin in EDTA (25 mM in 50 mM NaCl, pH 7.4), and then plated in the inner 60 wells of 96-well plastic plates. The outer 36 wells were rimmed with sterile water. For the MTT assay, cells were

seeded at a density of 2.5×10^3 cells/well in growth media and allowed to attach in the wells for 48 h, at which point the cells were preconfluent (approximately 60% confluent) and cell density was 5.0×10^3 cells/well or greater. The growth culture media was then removed; the desired study factors or peptides were then added in 100 µl/well of serum-free media, i.e., M199 alone. Serum-free M199 alone was used for control. As the MTT metabolic bioassay requires on average a minimum of 48 h for detection (range 4–72 h), initially cells were incubated with the added growth factors for 48 h to 6 days to determine the optimal incubation timing. For incubations longer than 3 days, on the third day of incubation, the conditioned media was discarded and growth factors were re-added. Cells were re-fed with growth factors after 3 days, as prior studies [Hansen et al., 1989] have demonstrated that refeeding is necessary to maintain continuous linearity in the development of absorbance.

After the cells were incubated with SP or added growth factors for the desired time interval (6 days), MTT (100 µl/well) was added, to reach a final concentration of 1 mg/ml in each well, and incubated with the cells at 37°C for 2 h. Next, lysing buffer (100 µl/well) was added to each well and allowed to incubate with the cells overnight at 37°C. Absorbance was then determined at 600 nm with a 96-well Bio-Rad 3550 Autoreader (Hercules, CA) using MTT plus the extraction buffer as control. MTT stock solutions were prepared by dissolving MTT at a concentration of 5 mg/ml in sterile phosphate-buffered saline (PBS). Stock solutions were filter sterilized with a 0.2-µm filter and stored in a capped lightproof container at 5°C for up to 1 month prior to use. Lysing buffer was prepared by dissolving 20% w/v SDS at 37°C in a 50% solution of DMF and deionized water. The pH of the lysing buffer was adjusted to 4.7 by adding a mixture of 80% acetic acid and 2% 1 N HCl.

Human Platelet-Poor Plasma Preparation

Platelet-poor plasma (PPP) was prepared as previously described [Pledger et al., 1977]. Briefly, fresh (24–48 h), tested, recovered human plasma units (approx. 300 ml/unit) were obtained from the Sacramento Blood Bank. In order to avoid platelet rupture and associated growth factor release, the plasma was collected and handled with care to minimize agitation and avoid freeze-thawing. In addition, prior to processing, 4 ml/unit of proteolytic enzyme inhibitors (1 mM phenyl methyl sulfonyl fluoride, 100 mM 6-aminocaproic acid, and 1% v/v aprotinin) was added to each unit of plasma. The plasma was then centrifuged at 13,800*g* for 1 h at 5°C. A small RBC pellet was formed and discarded. Next, the plasma was recentrifuged and the supernatant heated for 30 min at 56°C to precipitate fibrinogen. A small precipitate was removed and the plasma centrifuged for a third time and then extensively dialyzed (MW cutoff: 3,500) against PBS overnight at 5°C. The dialyzed plasma was centrifuged once more and then passed through a 0.45-µm filter. Prior to use, the HPPP was bioassayed and shown not to stimulate the growth of sparse populations of Balb/c 3T3 fibroblasts (kindly provided by W.J. Pledger), and not to differ significantly from control (M199 alone) when incubated with endothelial cells in concentrations of up to 15%, with 20% FBS serving as a positive control.

Data Analysis

All experiments were performed in 3 wells per point. Results are expressed as the mean ± SEM of data from experiments performed in triplicate ($n = 3$). Data were analyzed by a two-tailed Student's t-test and significance determined at $P < 0.05$.

RESULTS

In bioassay systems, the MTT assay has been valuable for detecting the effects of lymphotoxins, interleukins, interferons, and growth factors, and is also widely used as a measure of cell proliferation [Loveland et al., 1992; Weichert et al., 1991]. The biochemical principle involved in the MTT assay is the detection of metabolic enzyme activity as indicated by the reductive conversion of tetrazolium bromide salt to formazan by actively growing cells. This reaction is catalyzed by various cellular dehydrogenases (e.g., glutamate, malate, and succinate dehydrogenase) and diaphorases. In eukaryotic cells, succinate dehydrogenase catalyzes the oxidation of succinate to fumarate and is one of the major regulatory enzymes of the tricarboxylic acid (TCA) cycle. The production of formazan by the MTT assay can be quantitatively measured spectrophotometrically after cell lysis. In this system, absorbance measures correlate with changes in cellular reducing capacity and with cell growth as determined by cell number. The MTT assay is therefore a useful assay for com-

parative assessments of cell growth and metabolism.

The advantages of the MTT assay over other methods are multiple. Specifically, the MTT assay allows for highly reproducible, rapid, objective, low-cost, nonradioisotopic measures of subtle changes in cellular metabolic function in physiologically relevant conditions [Musser and Oseroff, 1994]. In addition, in contrast to tritiated radiolabeling, which indicates the number of cells synthesizing DNA, the MTT assay measures cellular reducing capacity of living cells and therefore provides additional information regarding cellular metabolism, as compared to conventional proliferative assays. In the present study, the relationship between endothelial cell number and absorbance was examined with MTT for the relevant range of cell concentrations. The results (Fig. 1) demonstrated a strong positive correlation ($r = 0.97$) in the two parameters. In contrast, control wells yielded mean background absorbance units of 0.0005, which was calculated to correspond to the activity of approximately 29 cells by extrapolating from Figure 1. However, as our results will demonstrate, in our endothelial system, the relationship between absorbance measures and cell number is complex when SP is used as the stimulus and indicative of effects in addition to those conventionally attributed to cell growth alone.

We have adapted the MTT assay for quantification of SP bioactivity in an in vitro endothelial cell culture system. The modified method [Hansen et al., 1989] used in the present study has identified and targeted critical laboratory parameters involved in the technique (pH and composition of the extraction buffer, final concentration of MTT, cell seeding density, and incubation time), which have led to a marked improvement in the sensitivity and reproducibility of results as compared to the original method [Mosmann et al., 1983]. A direct comparison with previously published methods has demonstrated that the modified method used in this study would yield 100% higher signals without concomitantly increasing the background signal.

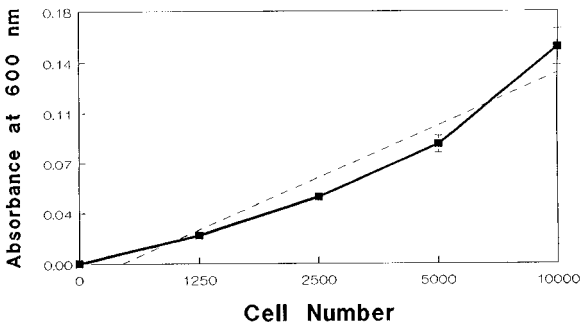

Fig. 1. Plot of absorbance at 600 nm as a function of cell number using the MTT assay study conditions (see text). The solid dark line (-■-) represents the curve obtained from data values (mean ±SEM, n = 3) and the dashed line (---) depicts the best linear curve fit for the data. The correlation coefficient (r) was 0.97.

In the present study, particularly careful attention was given to establishing appropriate cell seeding density and incubation timing due to the importance of these parameters in conducting the assay, as described above. Accordingly, we constructed absorbance/time-response curves for various cell seeding densities using 20% FBS as the growth stimulus. Our findings (Fig. 2) indicated that a cell seeding density, which resulted in 5,000 cells/well, or slightly greater for the MTT reaction, as that chosen for our study did, was optimal for absorbance linearity. In addition, 6 days of incubation with the study factors (with refeeding after the third day) was chosen as the incubation timing for all experiments, as no changes in absorbance at 600 nm were observed in endothelial cellular reducing capacity after 3 days, but by 6 days a reproducible difference in absorbance was appreciable at the selected range of cell concentrations (Fig. 2).

Previous studies from our laboratory have indicated that SP has growth-promoting effects on quiescent endothelial cells in serum-free conditions [Villablanca et al., 1994]. The effects, as measured by stimulation of ^{3}H-thymidine incorporated into DNA and cell counts, occurred after 48 h of incubation, were 10-fold greater than control and occurred at SP concentrations within the range of 10–500 µg/ml, with 150 µg/ml of SP having a significant consistent stimulatory effect. Importantly, SP synergized with insulin and human plasma factors in the growth response. Synergism between SP, insulin and plasma in the growth response was seen at SP concentrations of 0.1 µg/ml.

In contrast to our previous studies with SP on endothelial cell proliferation, as reported above, SP-stimulated changes in endothelial cell metabolic activity, as determined by cellular reducing capacity, occurred after longer incubation periods (6 days vs 48 h) and at lower SP concentrations (10 pg/ml vs 0.1–150 µg/ml) than SP-

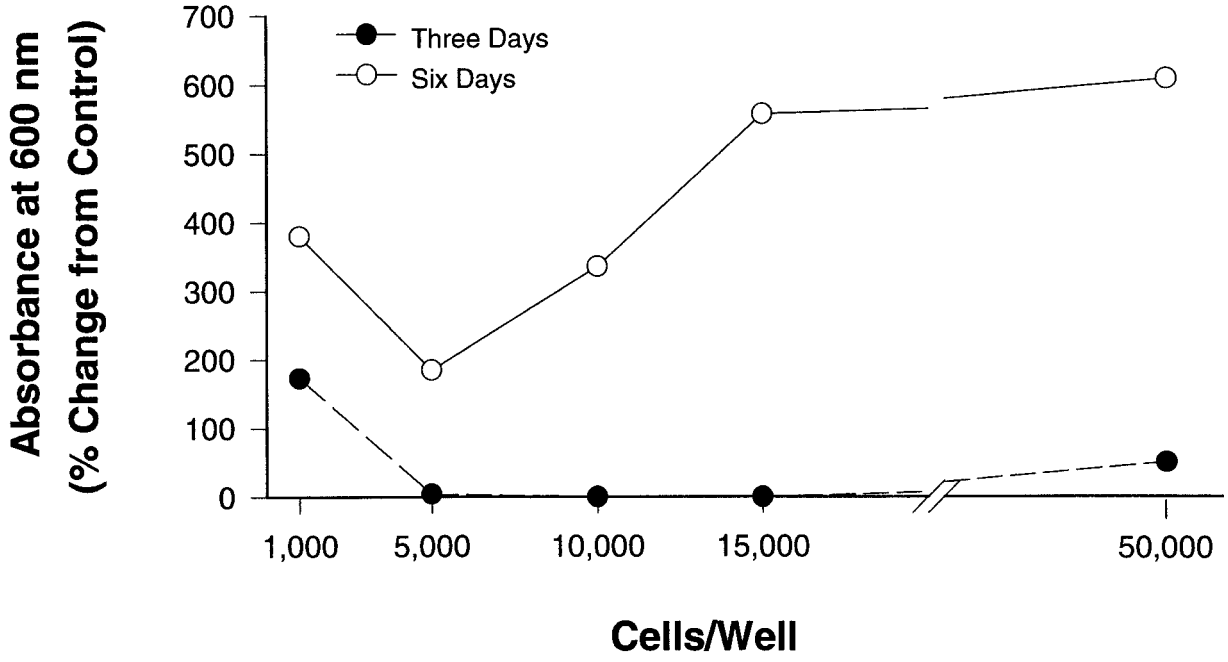

Fig. 2. Absorbance measures at 600 nm for various concentrations (1,000–50,000 cells/well) of endothelial cells in response to 20% FBS expressed as percent change from control, (M199 media alone). Data are shown for 3-day incubations (-●-) and 6-day incubations (-○-) with re-feeding after the third day as described in methods.

stimulated effects on DNA synthesis and cell growth. In addition, when incubated with endothelial cells, SP by itself (10 pg/ml–25 μg/ml) did not stimulate reducing capacity (Fig. 3A–C). In fact, SP alone was inhibitory and the inhibitory changes were significant ($P < 0.05$) when compared to blank (control) throughout the range of SP concentrations tested.

We subsequently wished to investigate whether factors in the cellular environment important in SP-stimulated cell growth might also modulate the effects of SP on cellular metabolic activity. Insulin has been demonstrated to potentiate the effects of other growth factors and to synergize with them [Villablanca et al., 1994; Woll and Rozengurt, 1989] for example, in the setting of cellular proliferation. Therefore, we evaluated the possible effect of SP plus insulin on cellular reducing capacity to determine whether a similar response would be observed on cellular energy metabolism. For these studies, endothelial cells were incubated with SP alone, insulin alone, or SP + insulin, with M199 alone (blank) serving as control. After endothelial cells were incubated with SP (10 pg/ml–25 μg/ml) in the presence of insulin (10 μg/ml), cellular reducing capacity was markedly enhanced compared to SP alone. Although insulin alone was slightly inhibitory compared to blank, significant ($P < 0.05$) stimulatory effects were observed for SP + insulin, versus SP alone, at all SP concentrations studied (Fig. 3A). The mean stimulatory effect of SP plus insulin was twofold greater than the effect of SP alone when averaged over the entire SP concentration range (10 pg/ml to 25 μg/ml). The effect of SP + insulin was no more than additive at SP concentrations of 10 pg/ml–0.01 μg/ml. However, the effect of SP + insulin was significantly ($P < 0.05$) greater than additive (i.e., synergistic) at SP concentrations of $\leq$1.0 μg/ml. Thereafter, there was a falloff in the synergistic response at high concentrations of SP (>1.0 μg/ml).

Since cells in the normal cellular environment are exposed primarily to plasma constituents, we next performed studies on endothelial cells co-incubated with SP plus HPPP. For these studies, endothelial cells were incubated with SP alone, HPPP alone, and SP plus HPPP, with M199 media alone serving as control (blank). The effect of HPPP alone (5%) was not significantly different from control. As compared to SP alone, HPPP (5%) significantly enhanced the response to SP at all SP concentrations studied (10 pg/ml–25 μg/ml), Figure 3B. The mean stimulatory effect of SP + HPPP was threefold greater than the effect of SP alone

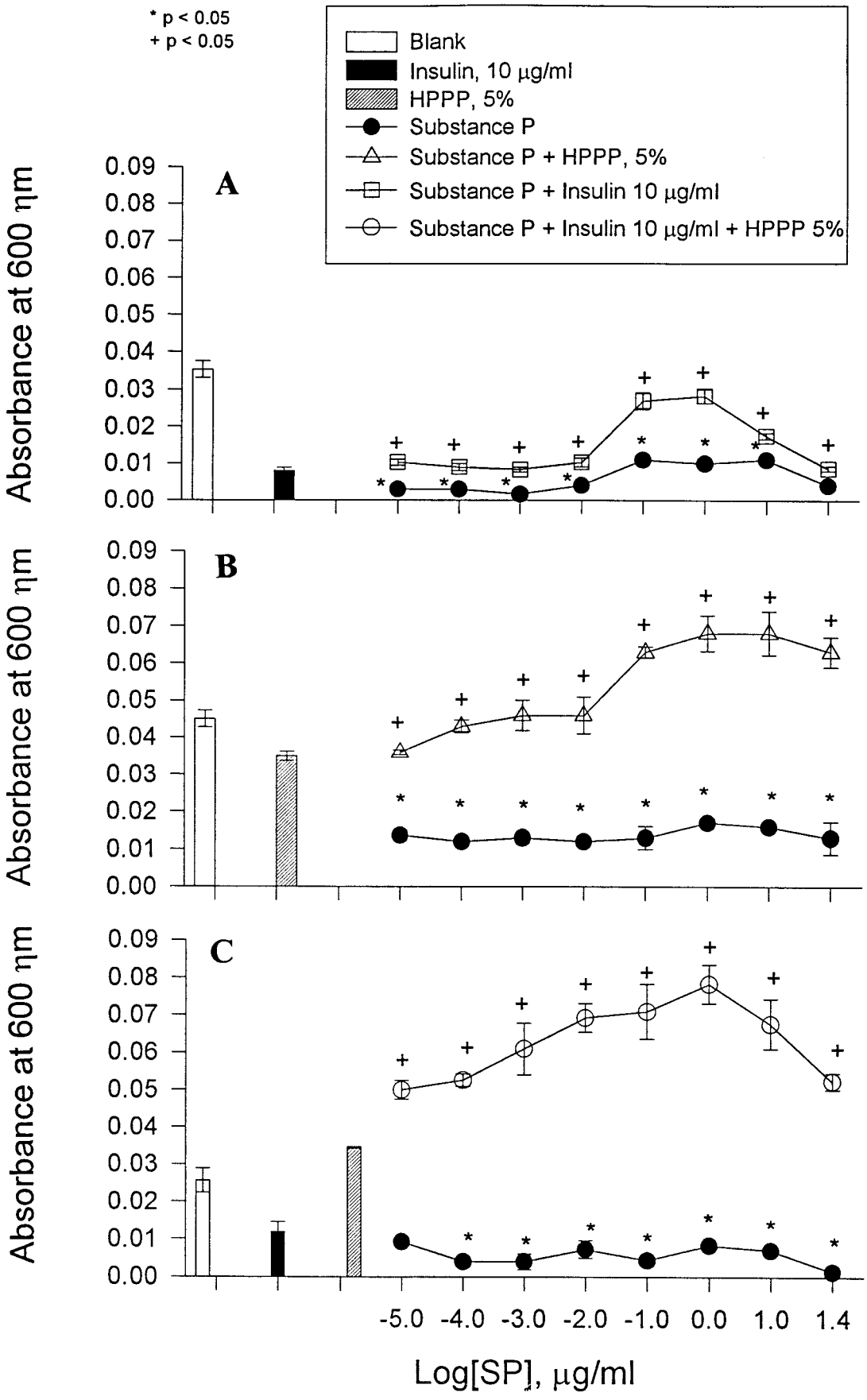
* p < 0.05
+ p < 0.05
Blank
Insulin, 10 μg/ml
HPPP, 5%
Substance P
Substance P + HPPP, 5%
Substance P + Insulin 10 μg/ml
Substance P + Insulin 10 μg/ml + HPPP 5%
A
B
C
Absorbance at 600 ηm
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
-5.0 -4.0 -3.0 -2.0 -1.0 0.0 1.0 1.4
Log[SP], μg/ml

Figure 3.

when averaged over the entire SP concentration range. The effect of SP + HPPP was no more than additive for SP concentrations of 10 pg/ml–0.01 μg/ul. Thereafter, the effect of SP + HPPP was significantly ($P < 0.05$) more than additive (i.e., synergistic) at SP concentrations of 0.1–25 μg/ml.

Lastly, in order to evaluate further the synergistic stimulatory interactions between insulin and plasma on endothelial cellular reducing capacity, endothelial cells were incubated with SP alone, and the response compared to SP + insulin (10 μg/ml) + HPPP (5%). M199 media alone (blank) was used as control. Insulin plus HPPP together (in the absence of SP) significantly stimulated cellular reducing capacity as compared to blank by 3.5-fold ($P < 0.05$, data not shown). Nonetheless, when compared to the response to SP alone, the combination of HPPP (5%), insulin (10 μg/ml) and SP had the greatest stimulatory effect on cellular reducing capacity. The effects were seen over the entire SP concentration range tested (10 pg/ml–25 μg/ml), Figure 3C. The mean stimulatory effect of SP + insulin + HPPP was 14-fold greater than the effect of SP alone when averaged over the entire SP concentration range. The response to SP + insulin + HPPP was additive only at 10 pg/ml SP. The response to SP + insulin + HPPP was significantly ($P < 0.05$) more than additive (i.e., synergistic) for all other concentrations of SP (0.0001–25 μg/ml SP).

In summary, as compared to SP alone, endothelial cellular reducing capacity was increasingly maximally stimulated by SP in the presence of insulin (10 μg/ml), HPPP (5%), and insulin + HPPP on average by two-, three-, and 14-fold, respectively. The response of SP + HPPP was on average 60% greater than the response of SP + insulin, and the response of SP + insulin + HPPP was on average fourfold greater than the response of SP + HPPP. In addition, the effect of SP combined with insulin, HPPP, or insulin + HPPP was at times greater than the additive effect of the components and significantly different than each component acting independently. The synergistic responses were dependent on SP concentration and were seen over the broadest SP concentration range when SP was in the presence of insulin and HPPP. These data are consistent with significant synergistic interactions between insulin and HPPP in stimulating endothelial metabolic activity in the presence of SP. The results imply an important role for these factors, singly or in combination, in modulating SP-mediated effects on endothelial cellular reducing capacity.

Fig. 3. Composite graph demonstrating effects of SP, insulin, and human platelet-poor plasma on endothelial cell reducing capacity. Graph demonstrates data for blank (B, medium 199 alone, open bar), SP alone (10 pg/ml-25 μg/ml, -●-), insulin alone (10 μg/ml, solid black bar), 5% human platelet-poor plasma alone (HPPP, hatched bar); and (**A**) SP plus insulin (-□-), (**B**) SP plus HPPP (-△-) and (**C**) SP plus insulin plus HPPP (-○-) on absorbance of endothelial cells at 600 nm (mean ±SEM, n = 3). Significance ($P < 0.05$) values are for comparisons of: (i) SP alone (-●-) versus blank (open bar), (*); (ii) SP alone (-●-) versus SP plus insulin (-□-), SP plus HPPP (-△-), or SP plus insulin plus HPPP (-○-), ($^+$); and (iii) the sum of the effects of SP alone (-●-) plus insulin alone (solid black bar), versus the combined effects of SP plus insulin (-□-); the sum of the effects of SP alone (-●-) plus HPPP alone (hatched bar), versus the combined effects of SP plus HPPP (-△-); and the sum of the effects of SP alone (-●-) plus insulin alone (solid black bar) plus HPPP alone (hatched bar), versus the combined effects of SP plus insulin plus HPPP, (-○-), (**).

DISCUSSION

Substance P is a vasoactive peptide that plays a role in a variety of functions relevant to the vasculature. SP has a number of specific metabolic effects, including activation of protein, RNA, and DNA synthesis. Our previous studies [Villablanca et al., 1994] demonstrated that SP is a stimulus for endothelial cell growth and replication. As mitogenic and proliferative activity are, in general, closely linked to cellular metabolic activity, in the present study we investigated the role of SP on endothelial cell reducing capacity using a previously well-defined colorimetric metabolic assay, the MTT assay. In contrast to the results of previous studies, including our own, which demonstrated endothelial cell proliferation in response to SP [Villablanca et al., 1994; Ziche et al., 1990], the current study demonstrated inhibition of endothelial metabolic activity by SP. As compared to the inhibitory effects of SP alone, physiologic concentrations of SP in the presence of insulin, HPPP or insulin plus HPPP resulted in significant stimulation of endothelial metabolic activity that was on average two-, three-, and 14-fold greater than the response to SP alone, respectively. Furthermore, insulin, HPPP, and insulin + HPPP were synergistic in stimulating endothelial reducing capacity over a broad range of SP concentrations.

The findings of the present study highlight the influence of factors in the cellular environ-

ment in modulating cellular responses. Of particular significance in this context were our findings demonstrating synergism between SP, insulin and HPPP. The stimulatory effects seen with the combination of insulin and plasma are of particular relevance because they represent culture conditions, which most closely approximated the physiologic cellular environment. The findings are in agreement with the precedent of previous studies [Hirata et al., 1988; Villablanca et al., 1994], which demonstrated synergism between SP, insulin and other growth factors for SP-stimulated cellular proliferation. The results of the present study demonstrate that SP also interacts with insulin and plasma factors to produce synergistic effects on cellular reducing capacity in endothelial cells. It is unknown which factor(s) in human plasma are responsible for stimulating SP-mediated metabolic activity. However, they are likely to be high-molecular-weight constituents as the HPPP used in these studies was prepared following dialysis at a molecular weight cutoff of 3,500. Additional contributing factors to facilitated stimulatory and synergistic responses to SP in endothelial reducing capacity could also pertain to the effects of insulin and/or plasma on such factors as response sensitivity, regulation of differential subcellular function targeting, or the generation of cellular oxides or antioxidants, as discussed further below.

Synergistic interactions between insulin, plasma and SP in our previous proliferation studies suggested the response was due to synergistic interactions between signal transduction mechanisms as endothelial cell growth stimulation was sensitive to SP receptor (NK-1) pharmacologic blockade. However, the data in the present study suggest that the effects of SP on endothelial metabolic activity have different qualities from those associated with stimulation of cell growth. Differential metabolic responses could be mediated indirectly or via NK-1 receptor subtypes. In this regard, SP receptor subtypes have been described [Flock et al., 1993] and intracellular effectors have been demonstrated to be differentially activated [Fong et al., 1991] by activation of SP receptor subtype isoforms. Mechanistic divergence in functional responses has previously been described in other cell types [Gauntt et al., 1994; Sung and Dietert, 1994] for other growth stimuli, such as, insulin [Okajima et al., 1992], and genistein [Pagliacci et al., 1993]. Therefore, precedent already exists for differential response targeting of a cell in response to a given stimulus.

Bioassay conditions that affect endothelial cell responses in the MTT assay depend on incubation timing. In this study, we observed that a longer time course was necessary for SP to elicit metabolic effects (6 days) than we had previously observed for effects on endothelial cell growth (2 days). These results are significant because they represent an effect of SP on endothelial cells that, although occurring over longer time periods, is in response to low and physiological concentrations of SP. In addition, the measured increases in reducing capacity suggest a temporal relationship between periods of maintenance of endothelial cell function (as opposed to proliferative activity) and the ability of SP to induce increased reducing capacity. Our findings also indicated inhibition of endothelial metabolic activity in response to SP alone, as evidenced by decreased absorbance compared to untreated cells. A number of factors, including cellular cytotoxicity, are reported to account for decreases in absorbance readings using the MTT assay [Jiang et al., 1993; Berridge and Tan, 1992; Pagliacci et al., 1993; Loveland et al., 1992; Huet et al., 1992; Okajima et al., 1992]. However, decreased MTT reducing capacity in response to SP in our system did not correspond to cellular toxicity, since it occurred under conditions where SP alone stimulates DNA synthesis and cell proliferation. Therefore, S-phase was temporally correlated with decreased MTT reducing capacity.

The MTT reaction is catalyzed by intracellular enzymes with reducing activity. Determinants of endothelial cell reducing capacity of particular interest to our findings, relate to nonmitochondrial reduction of nitrogen oxides. Although MTT reduction was initially thought to be linked entirely to mitochondrial dehydrogenases [Mathews and VanHolde, 1990], it has recently been demonstrated to occur primarily outside the mitochondria [Berridge and Tan, 1993]. Up to 20–30% of intracellular nonmitochondrial reduction of MTT can be due to oxides [Burdon et al., 1993]. Therefore, as used in the present study, the MTT assay detects contributions from nonmitochondrial electron donor mechanisms occurring in the cells. A possible contributor in this regard, particularly relevant to endothelial cells, is NADPH diaphorase, an NADPH-dependent enzyme involved with the

production of nitric oxide (NO) by nitric oxide synthase (NOS). Biochemical evidence and immunohistochemical studies suggest NOS and NADPH-diaphorase colocalize [Afework et al., 1992] and that NADPH diaphorase is NOS [Hope et al., 1991].

In endothelial cells, NOS leads to the production of NO from L-arginine [Palmer et al., 1988] and NK-1 receptor-mediated NOS activity is important in SP-stimulated production of nitrogen oxides. Endothelial NO production has been previously associated with SP-mediated angiogenesis, endothelial cell migration and growth [Ziche et al., 1994]. Recent evidence has implicated intracellular oxides generated by NADPH oxide-stimulated production of superoxide to be an additional pathway important in regulating cellular growth control [Irani et al., 1997]. Changes in intracellular generated nitrogen oxides may also be important in SP-stimulated changes in cellular reducing capacity in endothelial cells. Specifically, in response to SP, endothelial cells in cycle may be programmed to a lesser reducing capacity, and resting cells toward greater reducing capacity. This could be an indication of cell cycle-dependent changes in reducing capacity, which could include activities such as NO synthesis. Although the ability of quiescent versus cycling cells to produce NO has not been directly investigated in endothelial cells, it has been studied in other cell types. The effects of NO on the cell cycle in mouse macrophage-like cells indicated that after treatment with NO, G_0/G_1 phase increased and early G_2/M and S-phase decreased [Takagi et al., 1994]. Investigations of the time course of NO production on the proliferative activity of cultured hepatocytes have also demonstrated an inverse correlation between DNA synthesis activity and NO production [Obolenskaya et al., 1994].

Substance P alone could have pleitropic effects to increase NO production while decreasing cellular reducing capacity. This sequence of events has been previously demonstrated in a chick macrophage system where cellular reducing capacity was negatively correlated with the amount of NO produced [Sung and Dietert, 1994]. Studies of the effects of cytokine-stimulated NO production on energy metabolism in SMC indicate that production of NO in these cells was associated with inhibition of activities of the mitochondrial respiratory chain [Geng et al., 1992]. A similar inverse relationship between NO production and reducing capacity could be operative in our endothelial cell system.

Insulin and HPPP may result in a different response when added to SP, as compared to SP acting alone, in that the three factors acting together could lead to decreased NO production associated with increased reducing capacity. Limited data are available in the literature to address this possibility. Although a specific plasma protein has been hypothesized to serve as a reservoir for NO produced by the endothelial cell [Stamler et al., 1992], little is known about how other constituents of plasma may function to regulate cellular NO. Insulin alone has been demonstrated to stimulate NO production in human umbillical vein endothelial cells [Zeng and Quon, 1996]. However, umbillical vein endothelium is functionally different from aortic endothelium, and it is unknown whether insulin directly modulates NO production in arterial endothelium. The contributions of the activities of mitochondrial and extramitochondrial dehydrogenases, and NADPH-dependent NOS to MTT reduction in endothelial cells exposed to SP, and factors that synergize with SP, warrants further study.

In conclusion, the findings of our study are consistent with the postulate that the neuropeptide substance P plays a role in stimulating reducing capacity in endothelial cells in the presence of insulin and non-platelet plasma factors. This peptide may exert regulatory control at multiple levels in cellular metabolism, including the TCA cycle and the synthesis of nitrogen oxides. Differential regulation of endothelial function by SP is likely in modulating proliferative and metabolic responses; and even in metabolic responses, MTT reduction may be increased or decreased with SP under different conditions. Therefore, in addition to its previously well documented vascular functions and mitogenic actions, the findings of our study suggest a new role for substance P in regulating endothelial cellular reducing capacity.

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