Calcium- and superoxide anion-mediated mitogenic action of substance P on cardiac fibroblasts

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Kumaran, C., and K. Shivakumar. Calcium- and superoxide anion-mediated mitogenic action of substance P on cardiac fibroblasts. Am J Physiol Heart Circ Physiol 282: H1855–H1862, 2002; 10.1152/ajpheart.00747.2001.—Substance P is released from nerve endings in the heart under pathological conditions like ischemia, but its action on cardiac cells has not been investigated. This study tested the hypothesis that substance P is mitogenic to adult cardiac fibroblasts and delineated the underlying mechanism(s). Substance P, acting via neurokinin-1 (NK-1) receptors, stimulated cellular hyperplasia over a range of 1–10 mmol/l. It elicited no change in net collagen production, total protein synthesis, or cell protein content but increased 45Ca uptake and superoxide generation. EGTA, N-acetyl-cysteine, and superoxide dismutase attenuated the hyperplastic response to substance P. A combination of substance P and EGTA enhanced superoxide generation without an increase in DNA synthesis, showing that an increase in superoxide production does not result in hyperplasia when extracellular Ca2+ is chelated. Together, the data suggest that substance P may activate, via NK-1 receptors, a hyperplastic but not hypertrophic response in adult cardiac fibroblasts and that alterations in redox state and Ca2+ homeostasis may act in concert to mediate its mitogenic action.

THE HEART IS COMPOSED OF MYOCYTES and nonmyocytes, most of which are fibroblasts. For a long time, defective myocyte function was believed to be solely responsible for cardiac failure and very little attention was focused on the contribution of fibroblasts to the structural and functional integrity of the myocardium (36). However, whereas myocytes are terminally differentiated and lose their replicative ability soon after birth, fibroblasts retain their ability to proliferate in response to humoral and mechanical stimuli even in the adult heart. Consequently, cardiac fibroblasts play a central role in wound healing and myocardial remodeling under various pathological conditions. Modulation of cardiac fibroblast function has therefore attracted increasing attention in the past decade (36). Extensive studies (3) have been carried out on the effects of angiotensin II on fibroblasts following identification of a renin-angiotensin system in these cells. The emphasis on the renin-angiotensin system and its role in fibrogenesis has, however, nearly precluded investigations on the possible involvement of other growth factors in regulating myocardial fibroblasts.

The discovery that the myocardium is innervated not only by cholinergic and adrenergic nerves but also by peptidergic nerves that synthesize and secrete neuromodulatory peptides, such as substance P (SP) (37), raises the possibility that these neuropeptides may modulate myocardial metabolism under normal and/or pathological conditions. SP, a potent peripheral and coronary vasodilator (39), is believed to be involved in inflammation (18), tissue repair, and fibrosis (8). More recently, it has been shown that SP acts as a trophic agent in certain cell types, such as arterial endothelial cells (33) and skin fibroblasts (15). Although SP is released from nerve endings in the heart under pathological conditions such as hypoxia and ischemia (13, 32), no attempt has been made to understand its action on cardiac cells. Moreover, because SP immunoreactivity is marked in the connective tissue of the heart (35), myocardial fibroblasts may be particularly susceptible to its action. It is surprising therefore that regulation of fibroblast function by SP has not hitherto been investigated.

The present investigation had two objectives: 1) to test the hypothesis that SP is a stimulus for cardiac fibroblast proliferation, and 2) to delineate the mechanism(s) underlying the mitogenic action of SP on cardiac fibroblasts, with focus on the possible involvement of superoxide anion and Ca2+. The results of this study suggest for the first time that SP, acting via the neurokinin-1 (NK-1) receptor, is a mitogenic stimulus for cardiac fibroblasts and that concentrated increases in superoxide production and Ca2+ influx may contribute to its mitogenic action.

MATERIALS AND METHODS

All chemicals were purchased from Sigma and were of the best available grade. [3H]thymidine (specific activity, 18 Ci/mmol), [3H]phenylalanine (specific activity, 6.4 Ci/mmol),...
and $^{45}\text{Ca}$ (specific activity, 12.35 mCi/g) were obtained from the Bhabha Atomic Research Center, India.

**Isolation of Adult Rat Myocardial Fibroblasts**

Ventricular fibroblasts from adult Sprague-Dawley rats were isolated following the method of Eghbali et al. (4) with minor modifications. Briefly, the ventricular tissue was minced and subjected to repeated enzymatic digestions with a mixture of collagenase type IA, trypsin, pancreatin, and deoxyribonuclease. Isolated cells were preplated for 150 min and the unattached cells were discarded. The adherent cells were grown in Dulbecco’s modified Eagle’s medium (DMEM)-medium 199 (M199)-fetal bovine serum (7:2:1). Cells from passages 3 and 4 were used for the experiments, and their fibroblastic nature was confirmed by immunocytochemistry using antibodies against vimentin, factor VIII-related antigen, and desmin. The cultures were negative for factor VIII-related antigen and desmin, ruling out endothelial and smooth muscle cells, respectively, but positive for vimentin, indicating that they were fibroblasts (99% purity). Serum-deprived, subconfluent cultures were exposed to SP for 24 h. During the last 4 h, the medium was also used for the assays except where indicated otherwise. SP was used at a concentration of 10 μmol/l in all the experiments except for the dose response.

**Measurement of DNA Synthesis**

DNA synthesis was measured in terms of $[^3\text{H}]$thymidine incorporation into trichloroacetic acid-insoluble material, as described earlier (23). Briefly, serum-deprived, subconfluent cultures were exposed to SP for 24 h. During the last 4 h, the cells were pulsed with 2.5 μCi/ml $[^3\text{H}]$thymidine and processed for determination of acid-precipitable radioactivity.

**Determination of Cell Number**

Serum-deprived, subconfluent cultures were treated with SP for 24 h. The cells were then washed, detached with trypsin-EDTA solution, and resuspended in growth medium. Cell counts were performed with the use of a Neubauer counting chamber.

**Measurement of Total Protein Synthesis and Cell Protein Content**

Protein synthesis was measured in terms of $[^3\text{H}]$phenylalanine incorporation into trichloroacetic acid-insoluble material, as described by Schorb et al. (21). After serum deprivation, confluent cultures were exposed to SP and 2.5 μCi/ml $[^3\text{H}]$phenylalanine for 20 h. The cells were then processed for determination of acid-precipitable counts. Protein content per dish was measured by a modified Lowry assay (38).

**Measurement of Net Collagen Production**

Confluent cultures were serum deprived and exposed to SP for 24 h. Net collagen production (present in cell monolayer and medium) was determined by a hydroxyproline-based assay, as described earlier (34).

**Measurement of Superoxide Production**

Superoxide generation was measured in terms of nitroblue tetrazolium (NBT) reduction into formazan, as described by Siwik et al. (25) with some modifications. The cells were incubated for 4 h in Krebs-Ringer phosphate buffer, pH 7.4, containing 1.5 mmol/l NBT, with or without SP. After the assay, the cells were washed and color was extracted for spectrophotometric quantification at 490 nm.

**Measurement of Ca$^{2+}$ Influx**

$^{45}\text{Ca}$ uptake by cardiac fibroblasts was assayed as described earlier (7). Cells were preincubated for 10 min in 1.0 ml of HEPES-buffered physiological salt solution (HBSS) composed of (in mmol/l) 145 NaCl, 5 KCl, 1 MgCl$_2$, 1.2 CaCl$_2$, 5 HEPES, and 10 glucose (pH 7.4 at 37°C). $^{45}\text{Ca}$ influx measurements were initiated by adding 5 μCi/ml $^{45}\text{CaCl}_2$. After incubation of the cells for 0, 15, 30, and 60 min, 1.0 ml of ice-cold HBSS containing 2 mmol/l lanthanum chloride was added and the medium was discarded within 10–15 s. The cells were washed three times in HBSS containing 1.0 mmol/l lanthanum chloride for a total of 15 s and solubilized in 0.5 ml of 0.1 N NaOH. Radioactivity was measured by liquid scintillation spectroscopy. Kinetics of $^{45}\text{Ca}$ uptake in the presence of SP was determined by the inclusion of SP in the incubation buffer. The time $\theta$ value was subtracted from other time points.

**Statistical Analysis**

Statistical evaluation of data was by one-way ANOVA, followed by a Bonferroni test. A value of $P < 0.05$ was considered significant. The difference in $^{45}\text{Ca}$ uptake between the control and SP-treated groups at each of the time points was evaluated by Student’s $t$-test.

**RESULTS**

**SP Exerts Mitogenic Action on Adult Cardiac Fibroblasts**

As shown in Fig. 1, SP, at 10 μmol/l, increased incorporation of $[^3\text{H}]$thymidine into DNA by ~46% ($n = 20$). Moreover, it was observed that even in the absence of insulin and transferrin in the culture medium, SP had a stimulatory effect of comparable mag-

![Graph showing effect of substance P (SP) on $[^3\text{H}]$thymidine incorporation into DNA.](http://ajpheart.physiology.org/)
magnitude on $[^{3}H]$thymidine incorporation, indicating that SP exerts the effect even in the absence of other growth factors (data not shown). To ascertain whether the increase in DNA synthesis with SP reflects a proliferative response, cell count was performed after treatment with SP. Furthermore, the dose-response was studied to determine the concentration range over which SP exerts mitogenic effect on adult cardiac fibroblasts in vitro. As shown in Fig. 2, even a dose of 1 nmol/l SP produced a small but significant increase in cell number. The effect was more marked at higher concentrations of SP. Although not statistically significant, the effect with 10 $\mu$mol/l SP was higher than with 100 nmol/l SP and was therefore chosen for further experiments.

**SP Does Not Influence Total Protein Synthesis and Net Collagen Production**

To determine whether SP has any effect on total protein synthesis, the incorporation of $[^{3}H]$phenylalanine into proteins was measured in confluent cultures of adult cardiac fibroblasts. Figure 3 shows that SP had no effect on protein synthesis at 20 h, suggesting that SP does not exert a hypertrophic action on these cells. This was confirmed by a lack of effect of SP on cell protein content per dish.

Because cardiac fibroblasts are the main source of collagens, the effect of SP on net collagen production (collagen deposition) was determined. The cell monolayer and medium were pooled and used for determination of collagen-associated hydroxyproline content. Results presented in Fig. 4 show that treatment of adult cardiac fibroblasts with SP did not alter net collagen production by these cells ($n = 6$).

**SP Enhances Superoxide Production in Cardiac Fibroblasts**

Consistent with its postulated role in tissue injury and repair, SP has been shown to promote superoxide production in certain cell types (28, 29). Therefore, we designed experiments to ascertain whether SP increases endogenous superoxide production in cardiac fibroblasts and to examine whether superoxide mediates the mitogenic action of SP on cardiac fibroblasts. SP was found to enhance superoxide production consistently. In confluent cultures (Fig. 5A), after exposure to SP for 4 h, the increase was found to be $\sim 18\%$ ($n = 20$). However, in subconfluent cultures (Fig. 5B), treatment with SP for 24 h resulted in a 35% (about twofold higher; Fig. 5A) increase in superoxide gener-
Significant differences between each of these treatments, statistical comparisons by one-way ANOVA, followed by Bonferroni test, were made among three groups at a time: control, SP treated, and SP treated in presence of spantide, EGTA, or Ca\(^{2+}\) per se did not decrease cell number significantly (data not shown). This, in conjunction with the increase in \(^{45}\)Ca uptake in response to SP (Fig. 7), sug-

**Fig. 6. Effect of spantide, SOD, and N-acetyl-cysteine (NAC) on SP-induced hyperplasia.** To assess the effect of spantide on SP-induced fibroplasia, subconfluent serum-deprived cells were incubated with 10 \(\mu\)mol/l spantide in presence of SP for 24 h after pretreatment of the cells with spantide-EGTA for 15 min. SP-induced increase in \(^{3}H\)thymidine incorporation was abolished (Fig. 1). EGTA per se did not decrease cell number significantly (data not shown). This, in conjunction with the increase in \(^{45}\)Ca uptake in response to SP (Fig. 7), sug-

**Superoxide Anion Mediates Mitogenic Action of SP on Cardiac Fibroblasts**

Although SP increases superoxide generation in certain cell types, such an effect has not been linked to a mitogenic response. In our study, the antioxidant N-acetyl-cysteine abolished the increase in cell numbers in response to SP, suggesting that SP-induced cellular hyperplasia may be mediated by a redox-sensitive mechanism (Fig. 6). The inhibitory effect of SOD on SP-induced mitogenesis (Fig. 6) further confirmed the role of superoxide in the hyperplastic effect.

**Fig. 5. Effect of SP on superoxide production.** A: confluent cultures of cardiac fibroblasts were exposed to SP at 10 \(\mu\)mol/l \((n = 20)\) in Krebs-Ringer phosphate buffer spantide (KRPBS) for 4 h in presence of nitroblue tetrazolium (NBT). To assess the effect of spantide \((n = 20)\) and EGTA \((n = 20)\), cells were incubated with spantide at 10 \(\mu\)mol/l and EGTA at 1 mmol/l in presence of SP after pretreatment of the cells with spantide-EGTA for 15 min. Ca\(^{2+}\)-free incubation was in KRPBS without Ca\(^{2+}\) \((n = 4)\). Values are means \(\pm\) SD. To determine significant differences between each of these treatments, statistical comparisons by one-way ANOVA, followed by Bonferroni test, were made among three groups at a time: control, SP treated, and SP treated in presence of any of these agents. Control, \(n = 20\). B: subconfluent cultures were serum-deprived for 24 h and then exposed to SP at 10 \(\mu\)mol/l \((n = 4)\) with or without superoxide dismutase (SOD) at 200 U/ml \((n = 4)\) for 24 h. To determine significant differences between each of these treatments, statistical comparisons by one-way ANOVA, followed by Bonferroni test, were made among three groups at a time: control, SP treated, and SP treated in presence of any of these agents. Control, \(n = 16\); SP treated, \(n = 16\). \(^{a}\)Significantly different from control; \(^{b}\)significantly different from SP-treated cells.

**SP Enhances Ca\(^{2+}\) Uptake by Cardiac Fibroblasts**

Figure 7 shows the kinetics of \(^{45}\)Ca uptake by cardiac fibroblasts. SP enhanced \(^{45}\)Ca influx into cardiac fibroblasts by 21% at 30 min \((n = 8)\) and 170% at 60 min \((n = 8)\). In the untreated cells, there was a linear increase in \(^{45}\)Ca uptake for 30 min, followed by a dip, whereas in the SP-treated cells, the dip was not observed so that the difference between control and SP-treated cells was much higher at 60 min.

**Ca\(^{2+}\) is Involved in Mitogenic Action of SP on Cardiac Fibroblasts**

SP is reported to stimulate hydrolysis of phosphatidylinositol 4,5-bisphosphate with resultant changes in Ca\(^{2+}\) homeostasis in some cell types (14). We hypothesized that Ca\(^{2+}\) may be involved in the expression of SP effects on cardiac fibroblasts. When extracellular Ca\(^{2+}\) was blocked by EGTA, the SP-induced increase in \(^{3}H\)thymidine incorporation was abolished (Fig. 1). EGTA per se did not decrease cell number significantly (data not shown). This, in conjunction with the increase in \(^{45}\)Ca uptake in response to SP (Fig. 7), sug-

atation \((n = 4)\). The observed effect of superoxide dismutase (SOD) was consistent with the stimulatory action of SP on superoxide formation (Fig. 5B). The stimulatory effect remained unaffected when cells were exposed to SP under Ca\(^{2+}\)-free conditions (Fig. 5A).

**Superoxide Anion Mediates Mitogenic Action of SP on Cardiac Fibroblasts**

Although SP increases superoxide generation in certain cell types, such an effect has not been linked to a mitogenic response. In our study, the antioxidant N-acetyl-cysteine abolished the increase in cell numbers in response to SP, suggesting that SP-induced cellular hyperplasia may be mediated by a redox-sensitive
gusted a role for Ca\(^{2+}\) influx in mediating the mitogenic action of SP.

**SP Acts via NK-1 Receptors on Cardiac Fibroblasts**

Spantide, an NK-1 receptor antagonist, attenuated the increase in cell number in response to SP, showing that SP acts via NK-1 receptors on cardiac fibroblasts (Fig. 6). Spantide was also found to inhibit the stimulatory effect of SP on superoxide generation (Fig. 5A), confirming the involvement of NK-1 receptors in mediating this response.

**DISCUSSION**

SP, a member of the tachykinin family of neuropeptides, is present in capsaicin-sensitive unmyelinated primary afferent nerve fibers in peripheral organs and envelops the vasculature, with the highest density of SP-containing network in the aorta and vena cavae close to the heart (5). It is also localized in the adventitia and the medial-adventitial border of the vessel wall and is synthesized and released from arterial endothelial cells (13, 10). In the heart, SP is released from sensory nerve endings in response to ischemia (13, 32). Light-microscopic autoradiograms reveal that the SP binding sites occur within clusters of connective tissue cells (35), suggesting that these cells may be susceptible to SP activation. Therefore, it is possible that SP released from neurons in connection with tissue injury might, besides exerting vasodilatory effects, stimulate surrounding connective tissue cells to proliferate, and initiate a healing response in the heart. To test such a possibility, the present study examined whether SP exerts direct mitogenic action on cardiac fibroblasts. Although an in vitro model has obvious limitations, the use of isolated cultured cardiac fibroblasts permitted evaluation of the ability of SP to exert direct effects on cardiac fibroblasts in the absence of any complicating systemic effects such as increase in cardiac workload, inotropic mechanisms, or tissue metabolism.

The findings of the present study showed that SP elicits a hyperplastic response in vitro in adult cardiac fibroblasts, as evidenced by an increase in the incorporation of thymidine into DNA and cell number. Whereas the mitogenic action of SP on other cell types, including dermal fibroblasts (15), is mediated by the NK-1 receptor, the effect of spantide in the present study (Fig. 6) is the first demonstration of NK-1 receptor-mediated mitogenesis in cardiac fibroblasts. The magnitude of the mitogenic effect of SP was comparable to that reported with skin fibroblasts, in which ~18 and 38% of \(^{3}H\)thymidine-labeled nuclei were observed in response to SP at 1 and 10 \(\mu\)mol/l, respectively (15). The hyperplastic effect of SP on cardiac fibroblasts, although modest, was very consistent and was validated by different observations, such as increase in cell number and thymidine incorporation, dose response, and the abolition or attenuation of its action by the receptor antagonist, EGTA, N-acetyl-cysteine, and SOD.

SP did not have any effect on total protein synthesis or protein content per dish in confluent cultures (Fig. 3). SP may thus elicit a hyperplastic but not a hypertrophic response in adult cardiac fibroblasts. The neuropeptide did not influence net collagen production (Fig. 4). The lack of an effect of SP on net collagen production may reflect an incomplete response of the cells under in vitro conditions. In vivo, other factors may act on fibroblasts, after activation by SP, to increase collagen production. Consistent with our finding, SP was shown to enhance proliferation of pulp cells without an increase in the production of matrix proteins, and it was suggested that it might enhance proliferative activity without influencing functional activity (30).

A concentration of SP, higher than its plasma concentration, was required to elicit a proliferative response in cardiac fibroblasts. The dose response was reported to display a bell-shaped distribution for DNA synthesis in endothelial cells, requiring an SP concentration of 10–100 \(\mu\)mol/l for a growth-stimulating response (33). In rheumatoid synoviocytes, however, SP induced mitogenesis at 10–1 \(\mu\)mol/l (12). Thus the cell type in question and the experimental conditions employed may be determinants of the effective concentration of SP. In the present study, SP produced a small
but significant stimulatory effect on cell number at a concentration as low as 1 nmol/l but the effect was more pronounced at 100 and 10 μmol/l (Fig. 2). It may be noted that the cells were exposed to SP in the absence of other serum-derived growth factors. In vivo, a lower concentration of SP may act synergistically with other growth factors to exert more pronounced effects. It is also possible that fibroblasts are exposed to higher local concentrations of SP in vivo, especially under pathological conditions.

**Mechanism of Action of SP**

Ca^{2+} and superoxide are both involved in mitogenic action of SP on cardiac fibroblasts. SP has been shown to modulate Ca^{2+} homeostasis in many cell types (20, 28, 29). For example, in Chinese hamster ovary (CHO) cells expressing the SP receptor clone (CHO-SPR), SP induces Ca^{2+} entry through activation of cation channels. Furthermore, it has been suggested that d-myoinositol 1,4,5-trisphosphate [Ins(1,4,5)P₃] may regulate both Ca^{2+} entry and Ca^{2+} mobilization from intracellular stores in CHO-SPR cells (14). In neutrophils, SP stimulates the hydrolysis of PIP₂ into diacylglycerol and Ins(1,4,5)P₃ with a rise in intracellular Ca^{2+} (28). SP and related tachykinins have been shown to evoke Ca^{2+} signaling in cultured myenteric neurons by the influx of extracellular Ca^{2+} through L- and N-type Ca^{2+} channels. These signals are abolished on removal of extracellular Ca^{2+} or by the addition of Ca^{2+} channel blockers, lanthanum chloride, and nickel chloride (20).

In contrast, very little is known about regulation of Ca^{2+} homeostasis in fibroblasts and the role of Ca^{2+} in mediating the effects of growth factors on fibroblasts. The results of this study show that SP has a stimulatory effect on ⁴⁵Ca uptake by the cells (Fig. 7). To our knowledge, this is the first demonstration of agonist-induced enhancement of ⁴⁵Ca uptake by cardiac fibroblasts. Furthermore, it was found that chelation of extracellular Ca^{2+} using EGTA completely abolishes the stimulation of fibroblast proliferation by SP (Fig. 1), suggesting that the mitogenic effect of SP is dependent on Ca^{2+} influx from the extracellular space. Thus our data show that changes in Ca^{2+} homeostasis may play a role in the stimulation of cardiac fibroblast proliferation by SP.

SP has been shown to stimulate superoxide production in synovial cells (29) and human neutrophils (28). Tanabe et al. (29) have reported that NK-1 receptor/phospholipase C-linked diacylglycerol formation and activation of protein kinase C is the signal transduction pathway for SP-stimulated oxyradical production in synovial cells. Liberation of free radicals in gastrointestinal tissue as part of an inflammatory reaction in response to tachykinins has also been demonstrated (11). In the present study, SP consistently increased superoxide production in cardiac fibroblasts (Fig. 5). Exposure of subconfluent cultures to SP for 24 h elicited a more pronounced (about twofold higher) effect than exposure of confluent cultures to SP for 4 h (Fig. 5, A and B). The effect of SOD (Fig. 5B) confirmed the stimulatory action of SP on superoxide generation. Probing a role for extracellular Ca^{2+} in the enhancement of superoxide production by SP, fibroblasts were exposed to SP under Ca^{2+}-free conditions. It was observed that whereas the effect of SP on superoxide production remained unaffected in a Ca^{2+}-free medium, a combination of SP and EGTA had a more pronounced effect on superoxide generation than SP alone (Fig. 5A). However, it was subsequently found that EGTA and Ca^{2+}-free incubation per se enhanced superoxide production in adult cardiac fibroblasts (22). Thus it is not clear from these experiments whether the effect of SP on superoxide production is dependent on extracellular Ca^{2+}. It is intriguing that both SP (Fig. 5) and EGTA (22) stimulate superoxide generation, although the former enhances Ca^{2+} uptake and the latter chelates extracellular Ca^{2+}. The mechanisms underlying the effects of SP and extracellular Ca^{2+} on superoxide production may be different and need to be addressed.

The inhibitory effect of N-acetyl-cysteine and SOD on the mitogenic action of SP showed that SP-induced fibroplasia is mediated, at least in part, by reactive oxygen species (ROS). This is the first demonstration of the involvement of ROS in mediating the mitogenic action of SP and is in agreement with the current thinking that ROS exert critical signaling functions to regulate cellular growth in response to growth factors (9, 6). Platelet-derived growth factor (26) and thrombin (17) have been shown to stimulate ROS production in smooth muscle cells and suppression of ROS inhibited platelet-derived growth factor- and thrombin-induced mitogenesis in these cells. Furthermore, angiotensin II elicits a hypertrophic response in smooth muscle cells via the production of both superoxide and H₂O₂ (40, 31).

It has been reported that free-radical generation and alterations in Ca^{2+} homeostasis may be related in some cell types. Oxidants have been shown to stimulate Ca^{2+} signaling by increasing cytosolic Ca^{2+} concentration (27), suggesting a physiological role of ROS and oxidative stress in the regulation of Ca-induced signaling. Increase in intracellular Ca^{2+} was detected in response to H₂O₂ treatment of vascular smooth muscle cells (19) and, in endothelial cells treated with H₂O₂ (2), there was a transient release of Ca^{2+} from intracellular stores. Furthermore, a link between SP-induced superoxide production and Ca^{2+} flux has been suggested. Tanabe et al. (28) have reported that NK-1 receptor/G protein-coupled d-myoinositol 3-phosphate formation with resulting Ins(1,4,5)P₃-induced transient increase in intracellular Ca^{2+} concentration is the main signal transduction pathway for SP-stimulated O₂⁻ production in human neutrophils, although in synovial cells superoxide production was not found to be linked to Ins(1,4,5)P₃-induced Ca^{2+} mobilization (29). In the present study, whereas the mitogenic action of SP was dependent on superoxide production (Fig. 6), a combination of SP and EGTA enhanced superoxide generation (Fig. 5A) but without an
crease in [3H]thymidine incorporation (Fig. 1). The observations show that an increase in superoxide production in response to SP does not result in increased cell proliferation when extracellular Ca\(^{2+}\) is chelated. It appears, therefore, that SP-induced fibroplasia may depend on both Ca\(^{2+}\)- and superoxide-sensitive pathways and that blocking either of these would abolish the proliferative response. Thus, whereas the literature points to the link between ROS and Ca\(^{2+}\) homeostasis in certain cell types, our results suggest for the first time that SP-triggered Ca\(^{2+}\) and oxidant signaling pathways may, in addition, be functionally coupled to a hyperplastic response in cardiac fibroblasts.

**Model of SP-induced cardiac fibroblast proliferation.** SP is reported to exert its mitogenic action via its G protein-coupled NK-1 receptor and activation of extracellular signal-regulated kinases (ERKs) (1). The SP antagonist (d-Arg1, d-Trp5,7,9, and Leu11) inhibits both G protein-coupled receptor-mediated signal transduction and cellular DNA synthesis in Swiss 3T3 cells and the activation of ERK-1 and ERK-2, induced by G protein-coupled receptor agonists (24). Moreover, activation of ERK-1 and ERK-2 is required for DNA synthesis in cardiac fibroblasts (16). ERKs represent an important intracellular target of an altered redox environment and activation of ERKs by ROS has been implicated in the growth response of smooth muscle cells stimulated with growth factors (6). A model of SP-induced mitogenesis in cardiac fibroblasts, mediated by Ca\(^{2+}\)- and redox-sensitive pathways and activation of ERKs, is consistent with our data and recent literature and may provide a basis for future investigations.

In summary, a fibroproliferative response in the heart is important for recovery from injury, but factors that regulate fibroblast proliferation in the heart remain largely unidentified. Despite the exclusive reliance on an in vitro model, the present study suggests that SP may be an “activator” of a mitogenic response in cardiac fibroblasts and proposes that SP-induced alterations in redox state and Ca\(^{2+}\) homeostasis may act in concert to mediate its mitogenic action. The idea of a neural pathway of activation of cardiac fibroblasts is attractive and needs to be explored because it may be particularly relevant in conditions like myocardial ischemia that stimulate cardiac sensory nerve endings and promote increased local release of SP and other neuropeptides. Future investigations should delineate the Ca\(^{2+}\)- and oxidant signaling pathways, and any cross-talk between them, that seem to mediate the mitogenic action of SP on cardiac fibroblasts.

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