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THE ROLE OF PROTEIN KINASE C ON THE CONTRACTILE RESPONSES OF TESTICULAR CAPSULE AND SUPERFICIAL FASCIA TO HYDROGEN PEROXIDE

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Abstract

The present *in vitro* investigation had two primary aims: firstly to compare the responses of testicular capsule (TC) and cutaneous superficial fascia (SF) to hydrogen peroxide. Secondly to evaluate the role of protein kinase C (PKC) on these responses by use of bisindolylmaleimide1 chloride (BIM), a specific PKC inhibitor and phorbol-ester dibutyrate (PDBu), a specific PKC activator. Rat TC and SF samples (six for each experiment) were removed and suspended under 2 g tension in an organ bath and, after stabilization, the responses to 200 nM PDBu or to a cumulative concentrations (0.2 to 1.6 µM final bath concentration) of hydrogen peroxide, in the absence and presence of 2µM (BIM), were recorded. Both TC and SF produced contractions in response to PDBu and in a concentration-dependent manner to hydrogen peroxide. These contractions were inhibited by BIM. In conclusion, it seems that PDBu- and hydrogen peroxide-induced contraction in both SF and TC are mediated by activation of PKC. In addition, these findings suggest that macrophages, via release of hydrogen peroxide, may have an important role in mediating wound closure.

Keywords:

Bisindolylmaleimidel chloride, Hydrogen peroxide, Fibroblasts, Phorbol-ester dibutyrate, PKC, Superficial fascia, Testicular capsule**.**

Introduction

Wound healing is an orchestrated phenomenon of interaction between various cellular and chemical mediators. At cellular level, macrophages (1) and fibroblasts (2), as well as chemical mediators released from these cells, are among these factors that play important roles at different stages of this phenomenon.

When activated specifically or non specifically, macrophages release variable quantities, up to 10 µM of free-oxygen radicals (3, 4). Characteristically, the release of oxygen-derived free radicals in the external cellular environment seems to be mediated by a "respiratory burst". This release involves an increase in oxygen consumption and activation of hexose monophosphate shunt on the cell membrane of phagocyte cells (5). Furthermore, many of the macrophage functions are mediated by oxygen-derived free radicals, which include its direct bactericidal activity (4), or indirectly via macrophage-inflammatory protein 1-α, a C-C chemokine having chemoatractant capability for macrophages (6). Macrophages also influence wound healing

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directly by secreting growth factors that stimulate proliferation of fibroblasts and collagen synthesis (7).

On the other hand, both superficial fascia (8) and testicular capsule (9), two connective tissues known to contain primarily fibroblasts, are employed in study of pharmacology of wound healing (2). Previous studies showed that these tissues have special pharmacological properties different from those reported for smooth muscles (9, 10,d 11).

For example, normal subcutaneous fascia doses do not respond to two classically recognized smooth muscle cell agonists namely acetylcholine or barium chloride (9), while it responds to the antihistamines mepyramine and diphenhydramine (9, 11).

To our knowledge, no previous *in vitro* pharmacological study has been conducted with the specific aim of testing the effect of hydrogen peroxide, at a non-toxic physiological concentration that is believed to be present during wound healing process (12).

Therefore, in this *in vitro* study, the responsiveness of testicular capsule (TC) and superficial fascia (SF), two fibroblast/myofibroblast containing connective tissues, were employed to answer the following two questions: firstly do these tissues models respond in a similar manner to hydrogen peroxide, an agent known to release free-oxygen radical.

Secondly, what is the role of PKC in these responses? For this purpose, we compared the responses of these tissues to PDBu and hydrogen peroxide in the presence and absence of bisindolylmaleimide I hydrochloride (BIM), a drug known to selectively inhibit PKC activation (13).

Materials and methods

Reagents

All chemical reagents employed were purchased from Sigma (UK).

Animals

Isolated connective tissues were taken from male, Hooded Lister rats (Bradford University strain) in the body weight range of 250 to 350g. All the rats were housed in plastic, clean sawdust-floored cages (59x38x22 cm) in groups of five. The sawdust and water were changed twice a week. They had free access to water and standard food pellets (CRM-P-Special Diet Services, Witham, Essex, UK).

Isolation and preparation of rat testicular capsule (TC)

Following the sacrifice of each animal with an intraperitoneally administered dose of sodium pentobarbital (200mg/kg) and cervical dislocation the testes were removed from the scrotum, and placed in a Petri dish containing aerated (95% O_2 and 5% CO_2) Krebs Henseleit solution. A small incision was made at one end and the seminiferous tubules and all the internal contents, including the blood vessels were very gently removed. A parallel cut was made at the other end, leaving a ring-like structure of capsular connective tissue, from which a circular strip (1x1.5cm) was chosen and mounted under 2g isometric tension in a 10 ml organ bath. This procedure was found to be an easy and a reproducible one for this preparation. One circular strip from each testicular capsule was used for each set of experiments bellow (n=5).

Isolation and preparation of the SF of the rat

Samples of the superficial fascia were always selected from the lower dorsal site. Using fine forceps, a thin piece of the exposed superficial fascia was pulled gently from the underlying adipose tissue of the skin and with aid of a blunt-ended scissors; a small incision was made from the edges extending about 3cm down the curvature of the body. A fat free, thin

transparent strip of 1x3cm was removed and immediately placed in Krebs Henseleit solution. Two comparable strips were removed from each animal and used separately for each set of experiments below (n=5). The resulting tissue strips of 1cm in width and 2cm in length were mounted under 2 g isometric tensions, in a 10 ml organ bath, for experimental purposes. During the stabilization period of at least 45 min, the tension was adjusted to 2 g and checked before initiation and at the end of the experiments. The tissue was bathed in Krebs Henseleit solution and maintained at 36-37^{o}C and bubbled continuously with a gas mixture of 95% O_2 and 5% CO_2.

Test of responsiveness of SF and TC to PDBu and hydrogen peroxide

The responsiveness of both SF and TC to 200 nM PDBu (0.1 ml of 20 µM stock solution in DMSO) and to a cumulative concentrations (0.2 to 1.6 µM, final bath concentrations, 20 to 160 µl) of 0.1 M hydrogen peroxide were recorded using an isometric transducer (Grass FT30C). Additions of each of the cumulative concentrations were made after attainment of plateau contractions which were occurred at approximately 5 min intervals. The responses were recorded on a Grass Polygraph (Model 7D, USA).

Test of the role of PKC on the contractile responsiveness of both SF and TC to PDBu and hydrogen peroxide

The aim of these experiments was to investigate the role of PKC in the contractile responses of both the superficial fascia and testicular capsule following exposure to hydrogen peroxide and PDBu. In order to verify this role, 2 µM (final bath concentration) BIM (in DMSO), was incubated with each tissue for 30 min prior to the test responses to cumulative concentrations (0.2 to 1.6 µM final bath concentrations) of hydrogen peroxide and to 200 nM PDBu. The final concentration of DMSO in the bath solution never exceeded 0.5%.

Statistical analysis

The data obtained were statistically analyzed using ANOVA, followed by Tukey post-hoc test or paired student t-test with $P<0.05$ was considered as the level of significance.

Results

Effect of BIM on the contractile responses of SF and TC to hydrogen peroxide

Hydrogen peroxide (0.2 to 1.6 µM) induced concentration-dependent contractions, which in the presence of 2 µM BIM the responses were almost completely abolished (Fig. 1). Prior incubation with BIM alone did not affect the tension and no responses were observed in these tissues. The maximum contractions that were recorded for FS and TC with the optimal concentration of 1.6 µM of hydrogen peroxide were 700 ± 65 and 638 ± 63mg which were significantly ($P<0.001$) reduced following prior incubation with 2 µM BIM to 60 ± 5 and 55 ± 6 mg (Fig. 1) respectively.

Effect of BIM on the Responses of SF and TC to PDBu

Both SF and TC produced contractions in a concentration-dependent manner. Overall, the contractions produced by PDBu on SF and SF were not significantly different. The control contractile responses to 200 nM PDBu induced were 547 ± 51 mg in rat testicular capsule and 586 ± 49 mg in rat superficial fascia ($P>0.5$). Following incubation with BIM, the contractions produced by PDBu were reduced significantly ($P<0.001$) in both SF and TC to 30 ± 4 and 35 ± 5 mg respectively (Fig. 2).

Discussion

In this study, the role of PKC in the mediation of the contractile responses of

fibroblasts was investigated. The results showed that direct stimulation of PKC, by use of PDBu, induced contractions in both the testicular capsules and superficial fascia. These findings suggest that PKC plays a role in the contractile responses in these tissues which known to contain primarily natural fibroblasts (9). This notion was confirmed by almost complete abolishment of these responses following prior incubation with bisindolylmaleimide I hydrochloride (BIM), a specific PKC inhibitor. Furthermore, the responses to hydrogen peroxide was found to be similarly abolished in the presence of BIM, suggesting that the mechanism of action of hydrogen peroxide were similarly mediated by PKC activation.

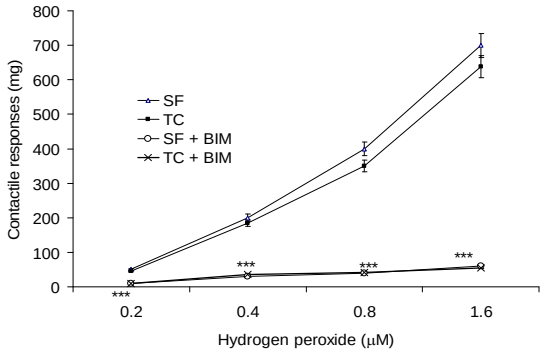

Fig. 1: Graphic presentation of dose/response curves of rat superficial fascia (TC) and testicular capsule (TC) preparations to varying concentrations of hydrogen peroxide (0.2 to 1.6 µM) in absence and presence of 2 µM bisindolylmaleimide1 chloride (BIM). BIM almost abolished the responses to hydrogen peroxide at all concentrations used (n=5, ***P<0.001 relative to control responses, ANOVA followed by Tukey post hoc test).

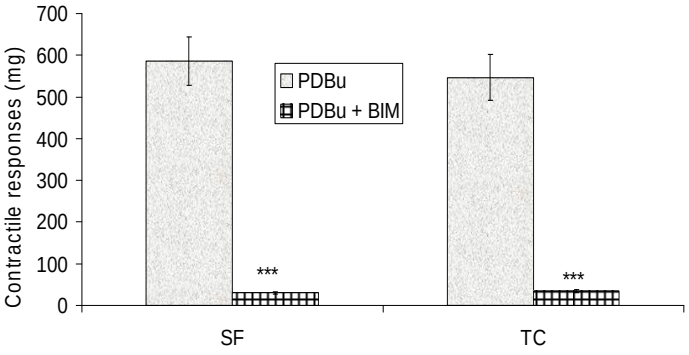

Fig. 2: A graphic representation of the contractile responses of rat superficial fascia and rat testicular capsule to 200 nM phorbol-ester dibutyrate (PDBu) in the absence and presence of 2 µM bisindolylmaleimide1 chloride (BIM). (n=5, ***P<0.001 relative to control responses, paired student t-test).

The presence of four PKC isoforms in rat embryo fibroblasts has been demonstrated earlier (14), and the present *in vitro* study supports these previous findings, and showed that PKC may have an important role in the wound healing process. These observations may have a degree of physiological significance since it is well documented that during many acute and chronic inflammatory responses and tissue injury the number of macrophages increases dramatically (7, 15). These cells are primarily responsible for debridement of dead tissues and phagocytosis of any foreign cells and bacteria in the wound area.

The mechanisms by which macrophages achieve this effect are believed to be mediated via release of reactive oxygen species (ROS) including hydrogen peroxide. If these free radicals, including hydrogen peroxide, escape from the confines of the cell due to overt cellular damage, they may be released into the wound area. These observations suggest a direct and previously unreported role of the macrophages, since besides their important and well established bactericidal action (16, 17), the released hydrogen peroxide may have another role in wound healing by inducing the contraction of myofibroblasts (2).

The observation that hydrogen peroxide induced a contraction is interesting, since it seems to have at least the double function of bactericidal action and induction of contraction of the myofibroblasts. The contractile responses to hydrogen peroxide were immediate, sustained, fully reversible, repeatable and concentration-dependent. All these features indicate that the effects produced were clearly not toxic to the myofibroblasts and may have physiological significance. Furthermore, the concentrations of hydrogen peroxide employed in this study were within the "normal" levels released by macrophages during wound healing process (4). The observed stimulatory effects produced by hydrogen peroxide on PKC are in agreement with the reported mechanism of action of this radical on nerve cells (18).

A recent study has shown that hydrogen peroxide produced contractions in isolated rat aorta that were mediated by ATP receptor activation (19). In our study, we did not attempt to elucidate the role of ATP receptor mediated actions on these tissues. Although this aspect needs to be assessed in a separate study, it is unlikely that the contractions observed in fibroblast-containing tissues are mediated similarly, since the responses in these tissues were sustained ones while those in the aorta were of transient nature.

A more recent pharmacological and electron microscopic study (20) has shown that the TC of various species (human, rat, mouse and rabbit), contains smooth muscles that contract ATP via activation of purinergic receptors, but invariable contract to ACh and noradrenaline. Although this study has demonstrated that TC contains smooth muscle cells, but the morphological features reported were described as flattened, diffused and elongated nature and not arranged in a compact layer as classically known for smooth muscles. It is possible that these cells could be fibroblasts for two reasons: firstly, since the fibroblasts also contain smooth muscle actin filaments and are contractile similar to smooth muscle cells (2, 10). Secondly, previous pharmacological studies have demonstrated that this tissue responds to smooth muscle agonists as well as two antagonists namely mepyramine and diphenhydramine (21).

What is the relevance of these findings in the understanding of the pharmacological principles that govern the wound healing process? Previous studies have shown that activations of PKC results in regulation collagen synthesis which is another important feature of wound healing process (15). In the present *in vitro* study we showed that PKC has an additional

role in promoting contraction of fibroblasts. The present findings have illustrated another function that macrophages play in the bewildering phenomenon of wound healing: it promotes contraction of tissues known to contain fibroblasts via its synthesis and release of hydrogen peroxide, an important function necessary for wound healing. Prudently, more studies are needed to assess the implications of these findings in clinical settings.

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