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Review

Role of Oxidants in the Signaling Pathway of Preconditioning

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ABSTRACT

This review focuses on the possible role of reactive oxygen species in the pathogenesis of this phenomenon. Evidence in support of a role of oxidants in preconditioning has come from the observation that administration of oxygen radical scavengers during the reperfusion period following the initial "preconditioning" ischemia could prevent the phenomenon. In addition, a brief exposure to a low, nontoxic dose of oxygen radicals may reproduce the beneficial effects of ischemic preconditioning, thus suggesting that radicals can directly trigger the preconditioning pathway. To explain the effects of oxidants in this setting, it has been suggested that reperfusion after the initial, "preconditioning" ischemic episode results in the generation of relatively low amounts of oxygen radicals, which are insufficient to determine cell necrosis, but nevertheless could modify cellular activities that have been implicated as mediators of the preconditioning phenomenon. Recent evidence suggests that low levels of oxidants may have a modulatory role on several cell functions. Possible mechanisms of oxidant-mediated protection might be protein kinase C and other kinases, ATP-dependent potassium channels, or changes in sulfhydryl group redox state, while an effect on adenosine metabolism, or the induction of myocardial stunning presumably does not contribute to oxidant-mediated preconditioning. Finally, *de novo* protein synthesis and gene expression, and increased antioxidant defenses might be involved in the late phase of preconditioning. In summary, available data strongly suggest that oxygen radicals might be possible mediators of preconditioning. However, further investigation is required to clearly elucidate their exact role and mechanisms of action. Antioxid. Redox Signal. 3, 3–10.

INTRODUCTION

THE PRECONDITIONING PHENOMENON was described for the first time in 1986 (41, 42). In their classic article, Murry and co-workers showed that the extent of necrosis following sustained ischemia could be paradoxically reduced if the ischemic period was immediately preceded by four brief episodes of ischemia, each lasting only 5 min. The protective effect was quite remarkable, as it compared favorably with reduction of infarct size as attainable by specific anti-ischemic interventions. Earlier studies showed that the preconditioning effect of ischemia was apparently short-lived, as protection was lost after 2 h. However, subsequent work has shown that protection could be regained after 24 h, and this phenomenon was thus termed "second wave of protection," or "late" preconditioning, to differentiate it from the "early" or "classic" preconditioning (31, 73).

Understandably, the preconditioning phenomenon has generated a broad interest, because the degree of myocardial salvage that could be achieved in this paradoxical way was the highest ever seen in studies on myocardial protection. Preconditioning has been reproduced in all animal species tested, and in organs other than the heart (31, 73). A large num-

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ber of studies tried to elucidate its mechanisms of action. Among the various hypotheses that have been advanced (31, 73), some have not been confirmed by subsequent works, whereas others have gained substantial evidence. The current view postulates that early preconditioning in the myocardium is initiated by release of several mediators (adenosine, bradykinin, norepinephrine, nitric oxide), which in turn activate an intracellular pathway of signal transduction, involving activation of protein kinase C and other kinases. The nature of the end effector of protection is still unknown; in the early phase of preconditioning, ATP-dependent K^+ channels are a reliable candidate for this role, whereas late preconditioning is actually viewed as mostly dependent on the synthesis of new proteins, with nitric oxide synthase as an important effector. However, the evidence available is still conflicting and largely debated.

This review focuses on the possible role of oxidants, and particularly oxygen free radicals, in the pathogenesis of this phenomenon. Oxygen free radicals are chemical species that, due to their very reactive nature, can oxidize and damage many cellular components. They form in little amounts during the normal metabolism of cells. However, when large amounts of oxygen free radicals are generated, such as during reperfusion of postischemic tissues, this can overwhelm cellular defenses, thus inducing tissue damage (9, 78). In addition to this well established toxic role when formed in large amounts, recent evidence suggests that low levels of oxygen radicals may have a modulatory role on several cell functions. This also is a paradox in and of itself, and it is befitting that oxygen radicals can be invoked to explain preconditioning.

ROLE OF OXIDANTS IN PRECONDITIONING

Evidence in support of a role of oxidants in preconditioning

A possible role for oxygen radicals in preconditioning was first suggested by Murry *et al*. (42), who showed that administration of superoxide dismutase (SOD), an enzyme that removes superoxide anions, during the reperfusion period following the initial "preconditioning" ischemia, could prevent the phenomenon. The authors hypothesized that myocardial reperfusion after the first, short ischemic episode results in the generation of low amounts of oxygen free radicals, not sufficient to determine cell necrosis, but which could modify cellular activities and thus induce preconditioning. Other investigators have subsequently obtained findings indicating that oxygen radicals may be involved in preconditioning. Administration of oxygen radical scavengers, such as mercaptopropionylglycine or SOD, was able to blunt the protective effect of ischemic preconditioning on infarct size (61, 63, 72). In another study, treatment with *N*-acetylcysteine negated the beneficial effect of preconditioning on postischemic recovery of contractile function (15). Similarly, the beneficial effect of preconditioning on reperfusion-induced arrhythmias was prevented by SOD administration during the preconditioning ischemia (46).

These studies, although strongly suggestive of a role for oxygen radicals in preconditioning, provide only indirect evidence. In this regard, it could be speculated that scavenger administration reduced overall cell injury, which in turn may have resulted in less activation of the preconditioning pathway(s). Direct demonstration that oxygen radicals do have the capability of triggering cellular mechanisms responsible for preconditioning has come more recently. In 1997, several other invesigators including us (67) and in Mobile (5), independently reported that a brief exposure of rabbit hearts to a low, nontoxic dose of oxygen radicals might reproduce the beneficial effects of ischemic preconditioning on infarct size as well as on recovery of left ventricular function (5,67). It should be stressed that this effect was elicited in the absence of ischemia. Thus, the data demonstrated that radicals can directly trigger the preconditioning pathway.

In addition to their role in the "classic" preconditioning, oxygen radicals have also been implicated as possible mediators of the "second wave" of protection. In fact, similar to what was observed with studies on "early" preconditioning, removal of oxygen radicals during

the preconditioning ischemia can also prevent the late development of protection against myocardial cell necrosis (58, 71, 72, 75), reperfusion arrhythmias (72), contractile dysfunction (57, 58), and vascular damage (28). Taken together, these data strongly support a role of oxygen radicals in the induction of both phases of preconditioning.

Evidence against a role of oxidants in preconditioning

Some studies, however, are in contrast with the previous observations involving oxygen radicals in preconditioning. Iwamoto *et al*. (26), in an *in vivo* rabbit model, administered SOD, alone or in combination with catalase, during preconditioning ischemia and subsequent reperfusion; in this study, the protective effect of ischemic preconditioning on infarct size was not prevented by scavenger administration. These findings are in striking contrast with the results obtained by Tanaka *et al*. (61, 62), who used the same animal and the same drugs, with opposite results. This discrepancy might be explained by the different preconditioning protocol: Iwamoto *et al*. used four cycles of preconditioning, whereas Tanaka *et al*. employed only one episode of preconditioning ischemia. It is conceivable that repeated cycles of ischemia might have resulted in greater generation of adenosine, enough to trigger preconditioning by itself even in the absence of oxygen radicals.

In another negative report, Richard *et al*. (50) could not prevent the beneficial effect of ischemic preconditioning with the use of monophosphoryl glyceraldehyde in rats subjected to coronary artery occlusion/reperfusion *in vivo*. To precondition rat hearts, several ischemic episodes must be used; moreover, many of the interventions that were effective in mediating or preventing preconditioning in other species were not effective in rats, thus suggesting that in these animals preconditioning may activate different signaling pathways.

In another negative study, Bassam *et al*. (6) used isolated rabbit hearts subjected to global ischemia and reperfusion, and SOD as the scavenging drug; the scavenger could not prevent the beneficial effects of ischemic preconditioning on recovery of myocardial function and lactic acid dehydrogenase release. However, in that study SOD was administered for the entire duration of the experiment. Thus, the design of the experimental protocol does not allow us to dissect the (beneficial) effects of oxygen radical scavenging during the preconditioning phase from the (injurious) effects of radicals during prolonged ischemic episode and the subsequent reperfusion.

MECHANISMS OF ACTION OF OXIDANTS IN PRECONDITIONING

Several mechanisms might explain the effects of oxygen radicals. Recently, it has become appreciated that exposure of cells to mild oxidative stress can reversibly modify several cellular activities, in the absence of cell damage, but secondary to changes in the activity of various enzymes and other cell components (2, 14, 16, 20, 49). Among others, oxidant stress can modify some of the cellular activities that have been implicated *in vivo* as mediators of the preconditioning phenomenon. It could thus be hypothesized that reperfusion after the initial, "preconditioning" ischemic episode results in the generation of relatively low amounts of oxygen radicals, insufficient to cause cell necrosis, but enough to modify cellular activities and thus induce preconditioning.

Protein kinase C and other kinases

A possibility that should be taken into account is the effect of radicals on protein kinase C. As many of the stimuli involved in preconditioning share the ability to activate this enzyme (31, 73), and as preconditioning can be prevented by inhibition of protein kinase C in various experimental models (55, 56, 74), activation of protein kinase C is currently held as a central mediator of ischemic preconditioning. With respect to the effects of oxygen radicals, studies in various cell types demonstrate that mild oxidative conditions can activate protein kinase C (11, 21, 33, 70). This effect is presumably linked to an increase of the Ca^{2+}/phospholipid-independent form of protein kinase C (21), and is accompanied by translocation of the inactive form of the enzyme from the cyto-

plasm to the cell membrane, where protein kinase C exerts its activity (70). The hypothesis that protein kinase C activation and/or translocation might be a mechanism of action of oxygen radicals in preconditioning received support by our observation that the preconditioning effect induced by oxygen radicals in the absence of ischemia was significantly attenuated by protein kinase C inhibition (67). However, as inhibition of protein kinase C did not completely prevent the beneficial effects of oxygen radicals, other mechanisms may be involved in this phenomenon. It has been suggested that important steps in the signaling pathway of preconditioning are represented by activation of phospholipase D (17, 66), tyrosine kinase (24, 48), and mitogen-activated protein kinase activation (38, 48), with complex interactions among these pathways that have not been entirely elucidated (12, 69). Again, oxidative stress has been proposed as a possible activator of these enzymes in various cell types (4, 7, 13, 25, 39, 44), and it is thus possible that an influence on these enzymes might contribute to oxygen radical-induced preconditioning, independent of protein kinase C activation.

ATP-dependent potassium channels

Oxygen radicals might also activate other cardioprotective mechanisms. In this regard, recent studies have shown that oxidants can increase the open state of ATP-dependent K^+ channels in patch-clamped myocytes (27, 64) and other cell types (34), and in isolated hearts (3). Opening of the ATP-dependent K^+ channels exerts a strong cardioprotective effect, and some studies have suggested that it might play a major role in the pathogenesis of preconditioning (23, 65). This possibility is supported by the preliminary observation that the preconditioning effect of hydrogen peroxide can be prevented by glybenclamide, an inhibitor of ATP-dependent K^+ channels (47).

Sulfhydryl group redox state

A main cellular target of oxidants is represented by sulfhydryl (—SH) groups of enzymes, which upon oxidation may react to form disulfide bonds (S—S), with subsequent alteration of protein structure and function. In isolated rat hearts, the beneficial effects of a brief ischemic episode on myocardial necrosis was prevented by agents that reduce —SH groups; in addition, preconditioning can be mimicked by administration of drugs that react with —SH groups forming disulfide bonds (77). These results suggest that ischemic preconditioning is associated with changes in the redox state of —SH groups, and it could thus be hypothesized that one mechanism for the preconditioning effect of oxygen radicals might be represented by their effects of —SH groups.

Changes in adenosine metabolism

Ischemic preconditioning can stimulate the activity of 5′-nucleotidase, the enzyme that releases adenosine from ADP. This phenomenon is accompanied by enhanced release of adenosine by ischemic myocytes, which in turn may exert cardioprotective effects (30). However, the evidence available suggests that this mechanism is unlikely to be activated by oxygen free radicals. In fact, lipid peroxidation, which is a consequence of oxygen radical generation (1), can *inhibit* brain 5′-nucleotidase *in vitro* (40), and administration of SOD, the enzyme that scavenges the superoxide radical, can increase adenosine release after coronary microembolization (60).

Induction of myocardial stunning

Another hypothesis on the pathogenesis of preconditioning considers myocardial stunning as a potential mediator. According to this theory, the brief ischemic episode would act by inducing stunning, which in turn would result in reduced energy consumption during the prolonged ischemia (29, 42). As myocardial stunning has been related to oxygen radical generation during reperfusion (10), it was hypothesized that preconditioning ischemia would act by inducing free radical generation, with consequent myocardial stunning, and myocardial protection during prolonged ischemia. However, several studies have shown that the beneficial effects of preconditioning are not related to myocardial stunning (22, 36, 43, 53). Thus, myocardial stunning remains an unwanted consequence of oxygen radical gen-

eration during ischemia/reperfusion, which presumably does not contribute to preconditioning. Finally, two other mechanisms have been involved as possible mediators of late preconditioning only, but they have not been implicated in the early phase of preconditioning.

De novo protein synthesis and gene expression

Expression of new proteins is considered to play a role only in the late phase of preconditioning, as 24 h are necessary for development of protection (31, 73). This has been confirmed by the observation that ischemic preconditioning causes a rapid increase in myocardial protein synthesis, which is necessary for development of late protection against myocardial stunning (51). A brief ischemic episode may increase the synthesis of several proteins, including heat-shock proteins, antioxidant enzymes, nitric oxide synthase, proto-oncogenes, a fatty acid transport protein, and several mitochondrial genes (18, 35, 37, 59, 76). Although the importance of nitric oxide synthase in delayed myocardial protection has been demonstrated (59), the precise role of most of these proteins in this phenomenon has not been elucidated (31, 62, 73), and there is still considerable controversy about the relative importance of antioxidant defenses (see below). It has been suggested that preconditioning may increase gene expression by influencing transcription factors. A brief ischemic episode is accompanied by activation of nuclear transcription factor-κB (NF-κB), and preconditioning may be prevented by NF-κB inhibition (38, 71).

Oxygen radicals play a major role in activation of NF-κB (54), and induction of the synthesis of heat-shock proteins (8, 45). In this regard, NF-κB activation during preconditioning is prevented by removal of oxygen radicals (10), and Kukreja *et al*. (32) have shown that exposure of isolated rat hearts to low concentrations of oxygen radicals results in increased expression of heat-shock protein mRNA.

Increased antioxidant defenses

As for the expression of new proteins, this phenomenon has been implicated only in the late phase of preconditioning. It has also been observed that exposure of hearts to a brief ischemic episode would result in increased antioxidant defenses 24 h later, which might in turn protect against reperfusion injury (31, 73, 75). These data are consistent with the hypothesis that part of the beneficial effect of myocardial preconditioning might represent a protection against the reperfusion-induced oxidative component of myocardial injury. It has to be pointed out, however, that previous studies have denied a role for increased levels of scavenging enzymes in preconditioning (19, 63, 68), and that the impressive beneficial effects of ischemic preconditioning cannot be related to this effect only.

In summary, available data strongly suggest that oxygen radicals *might be mediators* of preconditioning. However, further investigation is required to elucidate clearly their exact role and mechanisms of action, given the complexity of this phenomenon and oxygen radical biochemistry.

ABBREVIATIONS

NF-κB, nuclear transcription factor κB; —SH, sulfhydryl; SOD, superoxide dismutase.

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