characterized using sensitive preparations that discriminate between compounds with high and low affinities and between partial agonists and pure antagonists[15]. Kinins contribute to both contractile and secretory processes: the former are blocked by B_1 or B_2 receptor antagonists, while the latter tend not to be. Some secretory processes may therefore be activated by kinins through receptors other than B_1 and B_2 receptors (e.g. 'B_3'). Further developments in the area of kinin antagonists may help identify possible bradykinin receptor subtypes[18]; B_2 receptor antagonists also have therapeutic potential as analgesics or anti-inflammatory agents[19].

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Mergetpa: DL-2-mercaptomethyl-3-guanidinoethylpropanoic acid

Free radicals and ischemic tissue injury

Steven W. Werns and Benedict R. Lucchesi

There is growing evidence that reperfusion of ischemic organs is associated with the formation of free radicals that exacerbate the ischemic injury. Free radicals may damage viable tissue via the peroxidation of lipids and oxidation of protein sulfhydryl groups, leading to perturbations of membrane permeability and enzyme function. Steven Werns and Benedict Lucchesi *discuss evidence that activated neutrophils are an important source of free radicals after cardiac and intestinal ischemia, and assess the strategies that have been investigated as ways of alleviating damage caused by free radicals during ischemia–reperfusion.*

The early restoration of myocardial blood flow after the onset of myocardial ischemia is essential in order to arrest the progression of myocardial cell death and to permit the functional recovery of reversibly injured myocardium. There is, however, evidence that reperfusion of ischemic myocardium is also associated with the formation of oxygen radicals and that these radicals cause additional myocardial damage. A wide variety of cells, organelles and enzymes may be involved in the free radical formation activated by ischemia and reperfusion; these include neutrophils, xanthine oxidase, cyclooxygenase and lipoxygenase, autooxidation of catecholamines, mitochondria and the sarcoplasmic reticulum (see Refs 1 and 2 for review).

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Oxygen radicals

The sequential, univalent reduction of molecular oxygen results in the formation of superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH\cdot$) (Fig. 1). Superoxide anions and hydroxyl radicals are classified as free radicals by virtue of their unpaired electrons. The formation of hydroxyl radical from H_2O_2 can be catalysed by iron derived from ferritin, hemoglobin, or myoglobin[1].

Free radicals may exert diverse biochemical effects on both intracellular and extracellular molecules (Table I), and there is extensive evidence that cardiac structure and function can be altered by these effects. Perfusion of isolated hearts with a source of oxygen radicals causes a depression of contractile function accompanied by decreased myocardial concentrations of ATP and phosphocreatine; there is ultrastructural evidence of mitochondrial and endothelial damage[3]. Isolated

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TABLE I. Effects of free radicals on biological molecules

Target	Effect
Lipids	peroxidation of fatty acids, altering permeability
Proteins	oxidation of sulfhydryl groups, resulting in: activation of latent enzymes, e g. collagenase; inactivation of α_1-antitrypsin; inactivation of enzymes
DNA	strand scission, resulting in consumption of NAD and impairment of ATP synthesis

cardiac sarcoplasmic reticulum exhibited impaired Ca^{2+} transport after exposure to oxygen radicals[4].

Antioxidant mechanisms

A number of antioxidant mechanisms prevent oxidative damage by the reactive products of oxygen that are formed during normal metabolic events[1,2]. The primary intracellular defense mechanisms against this type of damage are superoxide dismutase (SOD), glutathione peroxidase, catalase, and vitamin E localized in the lipid membrane. SOD catalyses the formation of hydrogen peroxide from superoxide anions (Fig. 2). The decomposition of hydrogen peroxide to water and oxygen can be catalysed by the enzymes catalase and glutathione peroxidase (Fig. 2). The subsequent reduction of hydrogen peroxide or lipid peroxides by glutathione peroxidase is accompanied by the oxidation of glutathione, resulting in the formation of glutathione disulfide. The myocardial concentrations of free radical scavengers such as glutathione, glutathione peroxidase and superoxide dismutase are reduced by hypoxia, which may increase the susceptibility of the hypoxic cells to injury by free radicals during reoxygenation of the tissue[5].

The activities of SOD, catalase and glutathione peroxidase in the plasma are low or absent[1]. It has been suggested, therefore, that the primary extracellular defense against free radicals is proteins, such as ceruloplasmin, albumin and haptoglobin[1], and circulating erythrocytes[6]. Erythrocytes are effective inhibitors of damage caused by hydrogen peroxide, but not that caused by superoxide anions[6,7]. Reperfusion of ischemic, isolated rat hearts with erythrocytes increased ventricular function and decreased myocardial H_2O_2 (Ref. 7). Inhibition of erythrocyte catalase negated the protective effect, while inhibition of erythrocyte SOD did not. The results indicated that erythrocyte catalase can decrease endogenously generated H_2O_2 and related tissue injury.

Malondialdehyde and conjugated dienes are by-products of lipid peroxidation induced by free radicals. Several studies have found that malondialdehyde and conjugated dienes are formed during reperfusion of hypoxic or ischemic myocardium[5,8], although a recent investigation found no evidence of lipid peroxidation associated with reperfusion after global cardiac ischemia[9]. The reason for the discrepancy is unclear.

Detection of free radicals in the ischemic heart

The direct measurement of free radicals has been attempted using electron paramagnetic resonance (EPR) spectroscopy. Signals that are consistent with free radicals have been detected in the myocardium and coronary effluent of rabbit[10] and rat[11] isolated hearts during reperfusion after global ischemia. Spin-trapping techniques have been used to demonstrate the formation of free radicals during regional myocardial ischemia of the canine heart. EPR signals characteristic of oxygen and carbon-centered radical adducts were detected in the coronary venous blood draining from the ischemic bed by the infusion of a spin trap during reperfusion after a brief (15 min) period of coronary artery occlusion[12,13]. The production of free radicals by canine myocardium after 15 min of regional ischemia was markedly reduced by treatment with SOD plus catalase[12] or *N*-2-mercaptopropionyl glycine (MPG), a sulfhydryl compound that is believed to be a scavenger of hydroxyl radicals[13].

Myocardial infarction and free radicals

A variety of methods have been investigated to determine whether they can alleviate free radical damage during myocardial infarction.

Effects of free radical scavengers on infarction

The effect of superoxide dismutase and other free radical 'scavengers' on the extent of experimental myocardial infarction has been the subject of numerous studies (see Ref. 14 for review). In our laboratory, the effects of treatment with MPG, a scavenger of hydroxyl radicals, or the enzymes SOD and catalase, on the extent of myocardial injury have been examined in dogs that were subjected to 90 min of coronary artery occlusion followed by reperfusion. Treatment that began either 15 min before ischemia or 15 min before reperfusion was equally effective in limiting myocardial injury[15,16]. Subsequently it was found that treatment with catalase alone does not reduce infarct size significantly, while the administration of SOD alone limits the extent of injury in dogs that underwent coronary artery occlusion for 90 min followed by perfusion for up to 48 h (Ref. 14).

Tamura *et al.*[17] demonstrated a significant reduction of infarct size after 90 min of ischemia and four days of reperfusion in dogs treated with PEG–SOD (in which the polyethylene glycol conjugate

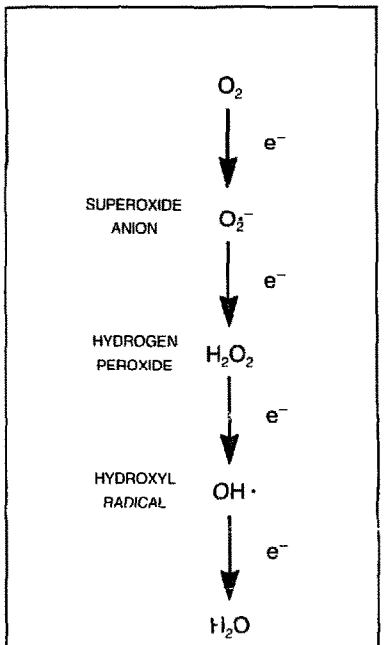

Fig. 1. Univalent reduction of oxygen. The sequential addition of single electrons (e^-) results in the formation of superoxide anion, hydrogen peroxide, hydroxyl radical and water.

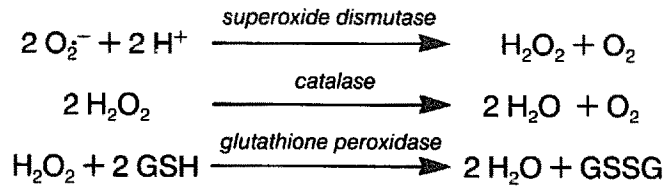

Fig. 2. Reactions catalysed by endogenous antioxidant enzymes. **GSH**, *glutathione;* **GSSG**, *glutathione disulfide.*

confers an extended plasma half-life). However, the efficacy of SOD has been challenged by other investigators who failed to observe a beneficial effect four or seven days after reperfusion in dogs undergoing coronary artery occlusion for 40 min or 90 min (Ref. 14). Tanaka *et al.*[18] reported that PEG–SOD does not limit canine infarct size after 90 min of ischemia and four days of reperfusion. In this study, the total dose was 10 000 U kg^{-1}, which resulted in a plasma concentration of 330 U ml^{-1} at the onset of reperfusion. In the study by Tamura *et al.*[17], the total dose was 1000 U kg^{-1}, which yielded a plasma concentration of 17 U ml^{-1} at the onset of reperfusion[17]. Paradoxically, the larger dose may have been responsible for the negative results obtained by Tanaka *et al.*[18] Omar *et al.*[19] observed that 5 mg kg^{-1} of human SOD significantly reduced infarct size in the rabbit, while a dose of 15 mg kg^{-1} did not limit infarct size, and a dose of 50 mg kg^{-1} significantly increased infarct size. Further work is required to explain the apparent toxicity of large doses of SOD. However, Ooiwa *et al.*[20] failed to observe a reduction in infarct size with PEG–SOD (1000 U kg^{-1}) in rabbit heart examined three days after occlusion–reperfusion. The authors raise the question of whether the discrepant results are due to species differences or to the method used to quantitate infarct size.

Effects of neutrophil inhibition or depletion on infarction

Results from several laboratories support the hypothesis that activated neutrophils are the primary source of oxygen radicals during the reperfusion of myocardium after prolonged regional ischemia. Rabbit or sheep antisera to canine neutrophils can be used to render dogs neutropenic. Dogs treated in this way exhibited less myocardial injury after 90 min of regional myocardial ischemia followed by reperfusion for up to 72 h than did dogs treated with saline or a non-immune serum[21].

A recently isolated monoclonal antibody, designated Mol 904 binds to the α subunit of both canine and human Mol, which is a heterodimeric adhesion-promoting glycoprotein expressed on the plasma membrane of neutrophils and mononuclear phagocytes[22]. The Mol 904 antibody does not induce neutropenia, and has been demonstrated to inhibit the binding of C3bi-opsonized particles and neutrophil aggregation. [Activation of the complement system results in opsonization of injured tissue by C3bi, which directs activated neutrophils to the injury.] Administration of the Mol antibody, or $F(ab')_2$ fragments of the antibody, beginning 45 min after coronary artery occlusion, significantly reduced infarct size in dogs undergoing 90 min of occlusion followed by reperfusion for up to 72 h (Ref. 22). These results provide additional evidence that myocardial injury during reperfusion may be reduced by suppressing the neutrophil-mediated injury that is superimposed on ischemic cell injury.

Inhibition of neutrophil function may explain the cardioprotective actions of a number of diverse agents, including adenosine, perfluorochemicals, ibuprofen, and prostaglandins such as PGE_1, prostacyclin and iloprost, a prostacyclin analogue[23,24].

Myocardial 'stunning' and free radicals

The delayed recovery of contractile function by viable myocardium after ischemia and reperfusion has been referred to as myocardial 'stunning'[25]. There is growing evidence that the formation of oxygen radicals during ischemia and/or reperfusion plays a role in the myocardial contractile dysfunction exhibited by myocardium injured 'reversibly' by a brief period of ischemia insufficient to cause necrosis (reviewed in Ref. 24). The most widely used experimental protocol to investigate the pathogenesis of 'stunned' myocardium involves the measurement of regional systolic wall thickening or segment shortening before and after occlusion of a canine coronary artery for 15 min.

Free radical scavengers

A variety of free radical scavengers have been reported to improve the recovery of myocardial function in dogs subjected to coronary artery occlusion for 15 min (Table II). Three independent laboratories have reported that the combination of SOD and catalase improved the return of wall thickening or segment shortening after 15 min of ischemia followed by reperfusion for 2 h or 3 h (Ref. 25). Dimethylthiourea (DMTU), a putative scavenger of hydroxyl radicals, and the low molecular weight sulfhydryl compound MPG enhance the recovery of 'stunned' myocardium[13,25]. Myocardial stunning was also attenuated by treatment with deferoxamine, which may prevent formation of hydroxyl radicals by chelating iron[25]. The beneficial effects of DMTU and deferoxamine have been interpreted as evidence for dysfunction caused by hydroxyl radicals. Deferoxamine, however, also may function as a scavenger of peroxyl radicals[26]. Recent data indicate that DMTU and deferoxamine may be capable of preventing injury by hypochlorous acid, a

TABLE II. Agents that attenuate myocardial stunning

- Deferoxamine
- Superoxide dismutase plus catalase
- Sulfhydryl compounds
  - *N*-2-mercaptopropionyl glycine
  - dimethylthiourea
  - *N*-acetylcysteine
  - captopril
- Xanthine oxidase inhibitors
  - allopurinol
  - oxipurinol

product of activated neutrophils[27,28], but there are conflicting data regarding the role of neutrophils in the pathogenesis of myocardial stunning (see below).

Until recently, the relative importance of ischemia and reperfusion in the development of post-ischemic myocardial dysfunction was unknown because the free radical scavengers had been administered before the onset of myocardial ischemia in all the studies of myocardial stunning. Bolli *et al.*[13] have now demonstrated that the administration of MPG either before coronary artery occlusion or immediately before the onset of reperfusion significantly improves the recovery of contractile function after 15 min of ischemia, whereas treatment beginning 1 min after reperfusion does not enhance the recovery of myocardial function. Production of free radicals is markedly suppressed by infusion of MPG immediately before reperfusion, while a burst of free radical production occurs during the first minute of reperfusion in the dogs receiving MPG 1 min after reperfusion. This suggests that most of the free radical injury responsible for myocardial stunning begins during the first minute of reperfusion.

Neutrophil inhibition or depletion

There is disagreement regarding the role of neutrophils in the pathogenesis of myocardial stunning. Treatment with iloprost, a prostacyclin analogue that inhibits the production of superoxide anions by activated neutrophils, increased systolic segment shortening after 15 min of ischemia and 3 h of reperfusion, while the myocardial function of dogs treated with an equihypotensive dose of sodium nitroprusside was similar to that of controls[29], and coronary perfusion with blood rendered agranulocytic by extracorporeal filters prevented myocardial stunning[25]. However, stunning was not prevented by a 90% reduction of the circulating neutrophil count in dogs treated with an antibody that had previously been shown to limit infarct size after 90 min of ischemia[25]. Furthermore, treat-

Does myocardial injury result from f

Activated species of oxygen generated by the enzyme xanthine oxidase have been postulated to cause cellular injury during reperfusion of ischemic tissue[1]. The role of xanthine oxidase in the pathogenesis of ischemic myocardial injury, however, is controversial. There is evidence that myocardial xanthine dehydrogenase, which does not generate oxygen radicals, is converted to xanthine oxidase during regional myocardial ischemia[1,2], but the effects of xanthine oxidase inhibitors on experimental myocardial infarction have been variable.

Treatment with allopurinol beginning at least one day before coronary artery occlusion limited the extent of canine myocardial injury after coronary artery occlusion for 60 min (Ref. 2) or 90 min (Ref. 3) followed by reperfusion for 4 h or 6 h, respectively. Dogs treated with allopurinol for 48 h before coronary artery occlusion exhibited less myocardial stunning than controls after regional ischemia for 15 min followed by reperfusion[4].

The beneficial effects of allopurinol appear to require a period of chronic pretreatment before coronary artery occlusion, since administration beginning either 30 min before coronary artery occlusion[5] or 15 min before reperfusion[6] did not reduce the extent of myocardial injury. The inefficacy of the acute treatment regimens suggested that the alleviation of xanthine oxidase-mediated myocardial injury by allopurinol, a competitive enzyme inhibitor, might require a longer treatment schedule that permits the metabolic conversion of allopurinol to its active metabolite, oxipurinol – a noncompetitive inhibitor with a longer half-life[7]. Therefore, it has been postulated that the accumulation of xanthine and hypoxanthine, the substrates of xanthine oxidase, within ischemic myocardium, might attenuate the inhibition of xanthine oxidase by allopurinol, while not affecting inhibition by oxipurinol[7].

Several studies have examined the effect of oxipurinol on canine infarct size. The administration of oxipurinol 15 min before and 3 h after reperfusion significantly reduced the extent of myocardial injury in dogs undergoing coronary artery occlusion for 90 min followed by reperfusion for 6 h (Ref. 6). Administration of a dose of oxipurinol before coronary occlusion or before reperfusion did not limit the ultimate extent of myocardial infarction in dogs subjected 40 min or 90 min of ischemia followed by one day or fo days of reperfusion[8–10]. This is consistent with previous da that indicated that short-term administration of a drug m merely delay tissue injury, while sustained therapy may required to prevent infarction. The infusion of iloprost, prostacyclin analogue, throughout the period of corona occlusion and the initial 2 h of reperfusion limited the exte of myocardial injury assessed after 6 h but not 72 h reperfusion[11]. Iloprost therapy that was continued until 4 after reperfusion, however, significantly reduced the exte of injury assessed 72 h after reperfusion[11].

One possible explanation of the conflicting results d scribed above is that low doses of allopurinol may increa reperfusion injury by reducing the concentration of u acid, which may act as a scavenger of free radicals. Zhong *al.*[12] recently reported the effects of allopurinol on exp imental liver damage caused by low-flow ischemia follow by reperfusion. Low doses of allopurinol actually increas cell injury, while higher doses of allopurinol prevented death completely[12]. The authors proposed that high conce trations of uric acid are required to prevent injury caused free radicals, so that partial inhibition of xanthine oxida by low doses of allopurinol could decrease the uric ac concentrations below that required to scavenge the sup oxide anions generated by the residual xanthine oxida activity.

Recent observations have provided reason to reconsi the hypothesis that xanthine oxidase plays a role in t pathogenesis of post-ischemic myocardial injury. First, th is evidence that the myocardial protection afforded treatment with either allopurinol or oxipurinol may not related to inhibition of xanthine oxidase. Allopurinol w reported to limit ischemic myocardial injury in both t rabbit and the pig, although xanthine oxidase activity w not detected in the myocardium of either species[13,14]. Al treatment with amflutizole, which is a more potent inhibi of xanthine oxidase than either allopurinol or oxipurin did not reduce either myocardial stunning or infarct size the dog, supporting the conclusion that allopurinol a oxipurinol may limit post-ischemic myocardial injury b

ment with the Mol antibody (which reduces infarct size after 90 min of ischemia) did not prevent myocardial stunning after 15 min of ischemia[30]. There was no significant accumulation of neutrophils during reperfusion of the myocardium after occlusion of a canine coronary artery for 12 min (Ref. 31), supporting the conclusion that neutrophils do not contribute to myocardial dysfunction after reversible injury. Thus, although one study has suggested that leukocytes cause the contractile dysfunction of reversibly injured myocardium, it appears that this dysfunction cannot be prevented by a clinically feasible approach such as the administration of a monoclonal antibody.

Xanthine oxidase inhibitors

Treatment with allopurinol improved systolic wall thickening after 15 min of regional ischemia, and oxypurinol enhanced regional contractile function after 90 min of coronary artery occlusion[25]. However, the effects of allopurinol and oxipurinol may be unrelated to xanthine oxidase inhibition, and there is controversy regarding the presence of xanthine oxidase activity in human heart (see Box).

Global myocardial ischemia and free radicals

Experimental preparations of global cardiac ischemia have been used as models of myocardial injury associated with cardioplegia during cardiopulmonary bypass or preservation for cardiac transplantation. Several experimental end-points have been used: left ventricular compliance and systolic function; tissue ultrastructure; the release of cytoplasmic constituents such as creatine kinase or lactate dehydrogenase; and the loss of metabolic substrates such as adenosine triphosphate and creatine phosphate. Each of the experimental end-points has been affected favorably by the administration of a free radical scavenger during global cardiac ischemia. In addition, EPR data have been interpreted as evidence for free radical production by isolated hearts after global ischemia[10,11], and deferoxamine enhanced the functional

icals produced by xanthine oxidase?

chanism unrelated to the inhibition of xanthine oxidase ivity[15,16]. Indeed, it has been suggested that the beneficial ects of allopurinol involve enhanced purine salvage[17].

On the basis of *in-vitro* data, it has been proposed that purinol serves as a scavenger of hydroxyl radicals[18] or pochlorous acid[19], an oxidant produced by activated trophils. Hypochlorous acid and its derivatives may mote tissue injury by the inactivation of α_1-antitrypsin, ich protects tissue elastin from hydrolysis by elastase[20]. ipurinol, but not allopurinol, has been observed to vent the inactivation of α_1-antitrypsin by hypochlorous d[19]. Treatment with allopurinol that was adequate to mit formation of oxipurinol, however, did not increase ability of feline plasma or lymph to scavenge hypoorous acid or inhibit lipid peroxidation induced by drogen peroxide plus myoglobin[21]. Thus, there are conting data regarding the capacity of allopurinol therapy to vide protection against oxidants.

The other reason for questioning the importance of thine oxidase is that biochemical assays have not tected xanthine oxidase activity in homogenates of human ocardium[22–24] (although immunohistochemical techues have been reported to demonstrate the enzyme's sence in the capillary endothelium of the human heart[25]). cently, it was reported that the human heart produced c acid during transient myocardial ischemia induced by onary angioplasty, indicating the presence of xanthine hydrogenase/oxidase activity[26]. Measurement of xanthine dase activity by chemiluminescence indicated the sence of substantial xanthine oxidase activity in human bilical vein endothelial cells[27], which also have been wn to release superoxide anions after hypoxia followed reoxygenation[28]. Thus, additional studies are needed to termine the mechanism of action of allopurinol and purinol, and further scrutiny of the xanthine oxidase ivity of human cardiac vascular endothelium may be rranted.

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recovery of isolated hearts after global ischemia[32]. Numerous other studies have described the beneficial effects of free radical scavengers on the function of rat, rabbit, dog and porcine hearts perfused with various crystalloid solutions before and after global myocardial ischemia.

Many investigators have proposed that the enzyme xanthine oxidase is the primary source of free radicals under such conditions. However, xanthine oxidase activity is either absent or is confined to the endothelium of rabbit, porcine and human hearts, and the effect of allopurinol on cardiac tissue may not be related to inhibition of xanthine oxidase (see Box). Thus, the free radicals produced by crystalloid-perfused hearts probably emanate from mitochondria, the sarcoplasmic reticulum, or other sources. It may be incorrect to presume that the hearts perfused with asanguinous solutions retain insufficient phagocytic cells to account for significant production of free radicals. A recent study concluded that the resident mast cells of the crystalloid-perfused rat heart are an important cause of injury during post-hypoxic reoxygenation[33].

□ □ □

The role of free radicals in the pathogenesis of myocardial stunning after reversible myocardial injury has been supported by numerous experimental studies. There have been inconsistent results, however, with respect to the ability of free radical scavengers to limit irreversible myocardial injury caused by prolonged myocardial ischemia. The conflicting data may relate to the differing experimental protocols and dosing regimens.

Despite the disagreement among experimental studies, preliminary results of two clinical trials were reported recently[34,35]. A randomized, double-blind, placebo-controlled, multicenter trial compared treatment with human recombinant SOD vs placebo in 114 patients who underwent coronary angioplasty for acute myocardial infarction[34]. Treatment with SOD did not alter the recovery of left ventricular ejection fraction or regional wall motion assessed by both contrast ventriculography one week after infarction and radionuclide ventriculography six weeks after infarction. This study has several deficiencies: inadequate sample size (if the true effect of SOD on the change in ejection fraction is an increase of 5% 170 patients would be required in order to achieve 90% statistical significance); the brief duration of therapy (1 h); and relatively late reperfusion (4 h after the onset of chest pain).

Forman *et al.*[35] reported an improvement in left ventricular function in patients with acute anterior myocardial infarction who underwent coronary angioplasty followed by intracoronary infusion of perfluamine (Fluosol-DA), a perfluorochemical that has been demonstrated to limit canine infarct size and inhibit free radical production by neutrophils. The results of only 11 patients were reported, however. Thus, further experimental and clinical studies will be required in order to prove that free radicals play an important role in the pathogenesis of myocardial infarction.

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Awards

Gilman wins Gilman

Al Gilman has been chosen as recipient of this year's prestigious ASPET prize established in honour of his father. The Louis S. Goodman and Alfred Gilman Award in Drug Receptor Pharmacology was awarded at this year's FASEB meeting in Washington in acknowledgement of Gilman's formative work on G proteins.