Activated epidermal growth factor receptor (ErbB1) protects the heart against stress-induced injury in mice

Miguel Pareja, Olga Sánchez, Jordi Lorita, Maria Soley, and Ignasi Ramírez

Department of Biochemistry and Molecular Biology, University of Barcelona, E-08028 Barcelona, Spain

Submitted 20 September 2002; accepted in final form 28 March 2003

Activated epidermal growth factor receptor (ErbB1) protects the heart against stress-induced injury in mice. Am J Physiol Regul Integr Comp Physiol 285: R455–R462, 2003. First published April 3, 2003; 10.1152/ajpregu.00588.2002.—Acute, high-intensity stress induces necrotic lesions in the heart. We found that restraint-and-cold (4°C) exposure (RCE) raises plasma lactate dehydrogenase (LDH), creatine kinase (CK), and transaminase activity in a time-dependent manner, with a peak value 7 h after stimulus cessation. At 24 h, signs of necrotic lesions were observed in paraffin sections stained with hematoxylin-eosin: focal accumulation of mononuclear cells in subendocardial areas of the left ventricle wall and focal hemorrhage in papillary muscles. In contrast, intermale fighting (IF) did not increase plasma CK activity, although LDH and transaminase activities did increase. In IF, no histological evidence of heart injury was observed. Because IF, but not RCE, increased plasma epidermal growth factor (EGF) concentration by ~1,000-fold, we hypothesized that EGF receptor (ErbB1) activation may protect the heart against stress-induced injury. To examine this hypothesis, we injected the ErbB1 tyrosine kinase inhibitor tyrphostin AG-1478 (25 mg/kg ip) immediately before mice were exposed to IF. After 3 h, plasma activities of LDH-1 and CK increased. Plasma enzyme activities were as low in control mice (injected with vehicle alone) as in nonfighting mice. In the last experiment, we injected EGF (0.25 mg/kg ip) 20 min before exposing mice to RCE. After 7 h, plasma LDH-1 and CK activities were significantly lower in these animals than in mice injected with vehicle alone.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
MATERIALS AND METHODS

Animals. Adult Swiss-CD1 male mice (Interfauna, Barcelona, Spain: 2.5–3 mo old, 35–40 g) were fed ad libitum and maintained under a constant 12:12-h light-dark cycle (lights on at 0800) and controlled conditions of humidity (45–55%) and temperature (22 ± 1°C). All experimental procedures were approved by the Committee on Animal Care of the University of Barcelona.

Restraint and cold exposure. On the day of the experiment (starting at 0900), mice were introduced into small flat-bottom cylinders (restrained) with adjustable head and tail gates (Panlab, Barcelona, Spain). This procedure resulted in an almost complete immobilization of the animals. Animals were maintained at 4°C for the indicated period of time and then killed (see below) or returned to individual cages at room temperature for recovery. During the recovery period, mice had free access to water and food.

IF. After the period of adaptation to the animal housing facility, mice were maintained for 15 days in individual cages (14.02 dm²). On the day of the experiment (starting at 0900), each mouse (the intruder) was introduced into the cage of an older (and larger) mouse (the resident) that had been isolated in a smaller cage (7.56 dm²) for ≥21 days. Residents had been tested for aggressiveness before the experiment. At the indicated time, the intruder was taken from the resident’s cage and killed (see below) or returned to an individual cage for recovery. During the recovery period, mice had free access to water and food.

Sampling and analysis. After the mice were anesthetized with pentobarbital sodium (60 mg/kg), blood was collected into heparinized syringes from the inferior vena cava. Blood plasma was obtained by centrifugation. A sample was deproteinized and neutralized as indicated elsewhere (24) and used for glucose quantification (59). Another sample was processed for EGF quantification as indicated elsewhere (22). LDH, α-hydroxybutyrate dehydrogenase (LDH-1), CK, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) activities in plasma were determined by standard procedures (LDH MPR1, HBDH MPR1, CK MPR1, AST MPR1, and ALT MPR1 assay kits, respectively, Roche, Mannheim, Germany). Plasma corticosterone was determined as indicated elsewhere (6). Immediately after collection of the blood, liver and submandibular salivary glands were excised, frozen in liquid nitrogen, and stored at −80°C until they were further processed (in < 1 wk). Submandibular glands were homogenized in 10 ml of PBS. After centrifugation (100,000 g for 60 min at 4°C), the supernatant was stored at −40°C for EGF quantification (22). Liver glycogen was determined as indicated elsewhere (24).

In some experiments, the heart was immediately excised after collection of the blood, fixed in 4% paraformaldehyde, and embedded in paraffin to obtain 7-μm-thick sections. The sections were stained with hematoxylin-eosin and used for histological examination.

Administration of EGF and/or tyrphostin AG-1478. In some experiments, animals were injected with tyrphostin AG-1478 (Calbiochem, Merck Eurolab; 25 mg/kg ip dissolved in 10% dimethyl sulfoxide) and/or EGF (Roche; 0.25 mg/kg ip dissolved in PBS) 20 min before the stress experience. Control animals received an identical volume of vehicles. After centrifugation (10 min, 1,000 g at 4°C), the supernatant was kept at −80°C until used for analysis, always in < 1 mo. Twenty-five micrograms of tissue protein [total extracellular signal-regulated kinase (ERK)] or 50 μg (phospho-ERK) were run in 12% SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA) using standard procedures.

Analysis of total ERK1/2. After transfer, the membranes were soaked in blocking solution (5% defatted powdered milk in PBS) for 60 min at 37°C, rinsed (5 times for 5 min each in 100 ml of rinsing solution (MTP): 0.5% defatted powdered milk and 0.05% Tween 20 in PBS), and incubated overnight at 4°C with the primary antibody [rabbit anti-murine mitogenic-activated protein (MAP) kinase (ERK1/2); catalog no. 06-182, Upstate, Barcelona, Spain] at 0.2 μg/ml in MTP. The membranes were rinsed as indicated above and incubated for 60 min at room temperature with the secondary antibody (GAR-IgG-HRP, Nordic, Tilburg, Netherlands; diluted 1:20,000 in MTP). After they were rinsed as described above, the membranes were developed with the ECL system (Amersham). Films were analyzed with Phoretix 1D Gel Analysis software after they were scanned (model GT-8500, Epson).

Analysis of phospho-ERK1/2. After transfer, the membranes were soaked in blocking solution (5% defatted powdered milk in Tris-buffered saline [TBS: 137 mM NaCl and 20 mM Tris, pH 7.6]) for 60 min at 37°C, rinsed (5 times for 5 min each in 100 ml of rinsing solution (MTT): 0.5% defatted powdered milk and 0.05% Tween 20 in TBS), and incubated overnight at 4°C with the primary antibody [mouse anti-murine phospho-MAP kinase (ERK1/2); catalog no. 05-841, Upstate] at 0.05 μg/ml in MTT. The membranes were rinsed as indicated above, incubated for 60 min at room temperature with the secondary antibody (GAM-IgG-HRP, Nordic; diluted 1:10,000 in MTT), and developed as described above.

Statistical analysis. To determine the significance of the differences in mean values, one- or two-way ANOVA and Tukey’s post hoc test were performed with SPSS software (SPSS, Chicago, IL). When no homogeneity of variances was detected (plasma enzyme activities and EGF concentration) by Bartlett’s test, logarithmic transformation was applied. If transformation did not homogenize variances, nonparametric Kruskal-Wallis one-way ANOVA was applied, and Dunn’s post hoc test was used.

RESULTS

We first studied the early responses to restraint- and cold exposure (RCE) and IF (Fig. 1). RCE and IF increased plasma corticosterone concentration by 13- and 20-fold, respectively. Although the mean values were higher in animals subjected to IF than in those subjected to RCE, the difference was not significant. We determined liver glycogen content, because one of the most important effects of circulating catecholamines is stimulation of liver glycogen breakdown. RCE and IF decreased liver glycogen content. The decrease was higher in RCE than in IF, but the difference between groups was not significant. RCE neither decreased the content of submandibular salivary gland nor increased plasma EGF concentration. In contrast, IF decreased submandibular salivary gland EGF content by >50% and increased plasma EGF by 600-fold.

Several hours are required for observation of tissue injury measured by the release of cytosolic enzymes to the blood (3, 48). Therefore, in the next experiment (Fig. 2), we determined plasma enzyme activities in samples obtained 0, 3, 7, or 24 h after animals were treated.
subjected to RCE or IF (60 min). Control animals (not exposed to stress) maintained constant activities of LDH, CK, AST (also known as glutamic-oxaloacetic transaminase), and ALT (also known as glutamic-pyruvic transaminase) in plasma. At the end of the stimulus, LDH, CK, AST, and ALT activities were increased in blood plasma of animals subjected to RCE. These activities continued to increase until 7 h after cessation of the stimulus and returned to control values by 24 h. At the end of the stimulus, in IF animals, LDH and AST activities were increased, CK activity was unchanged, and ALT activity was almost twice the control value, although the difference was not significant. LDH, AST, and ALT activities continued to increase until 3 h and decreased thereafter. CK activity was always the same as control.

Hearts from RCE and IF animals obtained 24 h after cessation of the stressful stimulus, as well as hearts from control animals, were processed for direct visualization of damage in the heart (Fig. 3). IF animals (Fig. 3B) showed no histological sign of necrotic lesions. The images were indistinguishable from those corresponding to control animals (Fig. 3A). In contrast, RCE animals showed focal accumulation of macrophage-like mononucleate cells surrounding damaged myocytes (Fig. 3C). Higher magnification revealed the lower eosinophilia of damaged myocytes (Fig. 3D). These alterations were observed in subendocardial areas of left ventricle wall. In some animals, focal hemorrhage was also observed in papillary muscles in the left ventricle (Fig. 3E and F).

Next, we analyzed the ability of tyrphostin AG-478 to block EGF action in whole mice. In this experiment, we studied one of the best characterized effects of EGF, the induction of ERK1/2 phosphorylation (62). Although we analyzed liver samples, because the liver contains the greatest number of EGF receptors in nontransformed cells (41, 53), this effect is not cell specific. At 10 min after EGF administration, bands corresponding to phosphorylated ERK1 and ERK2 appeared in liver extracts (Fig. 4). EGF also reduced the electrophoretic mobility of total ERK1 and ERK2. Simultaneous administration of AG-1478 abolished the effect of EGF. When AG-1478 was administered 1 h before EGF, the blockade was only partial. When it was injected 6 h before EGF, the inhibitory effect was completely lost.

A new experiment in which half of the intruder mice received an intraperitoneal injection of AG-1478 before they were introduced into the resident’s cage and the other half received vehicle was performed to study the involvement of ErbB1 in protection of the heart in the IF model (Fig. 5). IF increased plasma total LDH activity, but not activity of the cardiac isoform LDH-1 or CK. AG-1478 led to a further increase in plasma total LDH, LDH-1, and CK activities. Neither vehicle nor AG-1478 had any effect in nonfighting animals (data not shown).

In the last experiment, we addressed whether the lack of heart protection in the RCE model was due to the lack of EGF secretion from submandibular salivary glands. In this experiment, one-third of the mice received an intraperitoneal injection of EGF 20 min before they were subjected to RCE. Another third of the animals received combined doses of EGF and AG-1478. The rest of the animals were injected with vehicle. Samples were obtained 7 h after the mice were returned to their cages. RCE induced a large increase in plasma total LDH, LDH-1, and CK activities (Fig. 6). Previous EGF administration resulted in a significantly lower increase in total LDH, LDH-1, and CK activities. All these effects of EGF were abolished by simultaneous administration of AG-1478.

**DISCUSSION**

**RCE-induced cardiomyopathy.** Acute stressful stimuli of variable intensity induce necrotic lesions in the heart of several animal species (20). Here we provide histopathological evidence of necrotic lesions in the heart from RCE mice. Our observations, small foci of cellular infiltrates in subendocardial areas of damaged
myocytes in the left ventricle wall and hemorrhage in papillary muscles in this ventricle, coincide with other reports in rats (45), pigs (29), and rabbits (13). The appearance of mononucleate cells, similar to those observed after 24 h of the stressful stimulus, has been interpreted as part of the healing process (19). In a few days, these necrotic lesions in the ventricle wall evolve into nonspecific chronic lesions: accumulation of fiber and condensed fragments that correspond to remnants of myocardial cells (56).

The plasma enzyme profiles observed in RCE mice suggest heart injury. Thus we observed an increase in CK and LDH-1 activities. In addition, although both transaminase activities increased, the rise in AST activity was higher than the rise in ALT. Therefore, the ratio of AST to ALT increased (2.0 \pm 0.1 and 5.0 \pm 0.3 in control and RCE mice, respectively, \( P < 0.001 \)). None of these results are exclusive markers of heart damage but, taken together, strongly suggest a cardiac origin of these enzymes. Indeed, this is consistent with the histological results discussed above. Enzyme activities offer an easier and faster way to quantify the extent of tissue damage.

There is strong evidence that catecholamines are responsible for acute stress-induced heart injury. First, catecholamine administration induces necrotic lesions similar to those induced by stress (1, 13, 47, 54). Second, adrenergic, but not cholinergic, blockers reduce the extent of stress-induced heart injury as measured by enzyme activities in plasma (3). Some authors suggested the involvement of \( \alpha_1 \)-adrenergic receptors because of the effect of prazosin on norepinephrine-induced myocardial necrosis (31). Others pointed to \( \beta \)-adrenergic receptors on the basis of the effect of \( \beta \)-blockers (3) or isoproterenol (10, 57, 58). It is conceivable that both receptor types may be involved, and the balance between receptor signals would determine the final effect, as suggested for the effect of norepinephrine on apoptosis in cultured neonatal rat cardiac myocytes (28).

We propose that enhanced contractility (mediated by \( \beta \)-adrenergic receptors), in combination with \( \alpha_1 \)-adrenergic receptor-mediated coronary vasoconstriction (5), may induce functional hypoxia, which, together with accumulation of catecholamine metabolites (52), would result in sarcolemma damage, altered \( \text{Ca}^{2+} \) inflow, and, finally, cell death. This results in coagulative myocytolysis, which is the first morphological alteration to be observed after an intense catecholamine stimulation of the heart (58).

**Heart protection in fighting mice.** We reported previously that, after 3 h of cohabitation, plasma CK activity did not increase in intruder mice (48). Here we have extended the observation period to 24 h. The lack of rise in CK activity and the absence of necrotic lesions in the histological preparation suggest that the heart is protected in fighting mice. We did not find any reference in the MEDLINE database describing heart damage in rodents exposed to an aggressive male. At longer times, overcrowding appears to induce heart damage (17, 39, 60).

---

**Fig. 2.** Time course of plasma enzyme activities after RCE or IF stress. Mice were exposed for 60 min to RCE (stippled bars) or IF (solid bars). Control animals (open bars) were maintained in their cages. Then animals were returned to their cages with free access to food and water. At indicated times, animals were killed to determine plasma enzyme activities: lactate dehydrogenase (A), creatine kinase (B), aspartate aminotransaminase (C), and alanine aminotransaminase (D). Values are means \( \pm \) SE of 6 animals per group. Significant differences were determined by 2-way ANOVA, and Tukey's post hoc test was used to determine significance of differences vs. corresponding control: *\( P < 0.05 \); **\( P < 0.01 \); ***\( P < 0.001 \).
Aggressive behavior (isolation-induced fighting) is indeed a high-intensity stressor in rodents (30). We found that plasma corticosterone concentration was 20-fold higher in IF mice than in controls. This indicates a strong activation of the hypothalamus-pituitary-adrenal axis. In addition, liver glycogen content was decreased in IF mice. Glycogen degradation is an immediate effect of increased plasma catecholamine concentration. In rats, Sgoifo et al. (49) reported that defeat induced a higher increase in plasma catecholamine concentration than restraint. These authors reported that defeat resulted in a high risk of ventricular arrhythmias (49, 50). Therefore, the lack of heart injury in fighting mice may not be attributed to a lack of catecholamine stimulation of the heart. Rather, we suggest that, in this model, the heart is specifically protected.

**Does activated ErbB1 protect the heart?** Male mouse submandibular salivary glands accumulate as much as 30-fold more EGF than female glands (22). Because aggressive behavior induces acute secretion of EGF into blood plasma, and the result is a ~1,000-fold increase in plasma EGF concentration (40, 48), a role of...
EGF in response to this male-characteristic behavior was suggested but never proven.

To examine the role of the EGF receptor (ErbB1), we used the tyrphostin AG-1478, a highly specific inhibitor of ErbB1 tyrosine kinase activity. The IC₅₀ for ErbB1 is 100,000 times lower than that for related tyrosine kinases (HER2-Neu or platelet-derived growth factor receptor) (32). This inhibitor was shown to delay A431 tumor growth in nude mice (7) and to decrease hydroxyproline accumulation in a rat model of pulmonary fibrosis (46). In these studies, a dose of 50 mg·kg⁻¹·day⁻¹ was used. We observed that AG-1478, at 25 mg/kg, completely and reversibly blocked EGF-induced ERK1/2 phosphorylation in mouse liver. An alternative to AG-1478 would be the administration of neutralizing antibodies directed to ErbB1 or ErbB2 [the preferred partner for heterodimerization (62)]. To our knowledge, available antibodies are directed to human receptors, and they do not (or are not reported to) react with murine receptors (25, 37). We thus decided to examine the role of ErbB1, acutely activated by secreted EGF, by means of the effect of the ErbB1 inhibitor AG-1478.

We conclude that activated ErbB1 protects the heart against stress-induced injury, because 1) IF raised plasma CK and LDH-1 activities in AG-1478-injected mice and 2) activation of ErbB1 by exogenous administration of EGF greatly reduced the RCE-induced rise in plasma CK and LDH-1 activities. We showed previously that EGF (0.25 mg/kg ip) raised plasma concentration by ∼100-fold (18). The effect of exogenous EGF was dependent on ErbB1 activation, because it was completely blocked by simultaneous administration of AG-1478. Our results suggest that RCE mice were not protected against stress-induced heart injury because of the lack of EGF secretion from submandibular salivary glands.

Our results provide no insight into the mechanisms involved in the effect of EGF. However, several hypotheses can be outlined. If β- and α₁-adrenergic receptors are involved in catecholamine-induced heart injury, interference with signaling of either receptor would reduce the response to catecholamines and, hence, the effect of these hormones. We showed recently that EGF interferes with β-adrenergic signaling in heart myocytes and with the inotropic and chronotropic responses to epinephrine in perfused hearts (34). One consequence of these effects of EGF was protection against some of the harmful effects of catecholamines: the arrhythmogenic effect of sustained infusion of epinephrine in perfused hearts and induction of the Bezold-Jarisch reflex by repeated administration of epinephrine in whole animals (34). Whether the interference with β-adrenergic signaling also explains the protection of the heart against stress-induced injury remains to be established. In addition, a hypothetical effect of EGF on α₁-adrenergic receptor signaling will have to be explored. It has been reported that EGF interferes with contraction of norepinephrine-induced canine helical mesenteric arterial strips, an α₁-adrenergic receptor-mediated effect (38). However, interfer-

Fig. 5. Effect of AG-1478 on plasma enzyme activities in fighting mice. Mice were injected with AG-1478 (25 mg/kg ip) or vehicle alone immediately before they were introduced into a resident’s cage. Plasma enzyme activities in intruder mice were determined after 3 h. Values are means ± SE of 8 animals per group. Statistical comparisons were made by nonparametric Kruskal-Wallis 1-way ANOVA, and Dunn’s post hoc test was used to determine significance of differences between pairs of values: *P < 0.05; **P < 0.01; ***P < 0.001 vs. control. LDH, lactate dehydrogenase; LDH-1, α-hydroxybutyrate dehydrogenase; CK, creatine kinase.

Fig. 6. Effect of EGF on plasma enzyme activities in RCE mice. Mice were treated with EGF (0.25 mg/kg ip), with or without AG-1478 (25 mg/kg ip), 20 min before RCE for 60 min. Plasma enzyme activities in intruder mice were determined 7 h later. Values are means ± SE of 5 animals per group. Statistical comparisons were made by nonparametric Kruskal-Wallis 1-way ANOVA, and Dunn’s post hoc test was used to determine significance of differences between pairs of values: *P < 0.05; **P < 0.01; ***P < 0.001 vs. control.
ence with α1-adrenergic receptor signaling was not observed in other cell types (23).

Because catecholamine metabolites or very high oxidative products may participate in the initial steps of sarcolemmal damage, the protective effect of EGF may target oxidative stress processes. In many systems, EGF or other ErbB1 ligands were shown to be protective against oxidative-stress-induced damage or even cell death (2, 4, 9, 16, 33, 43, 61). Therefore, a number of possible mechanisms have to be explored to elucidate the molecular basis of the effect of EGF on stress-induced heart injury. Use of specific compounds to block different signaling pathways will require a new experimental model (perfused hearts or isolated myocytes), because, in the whole animal, these inhibitors would block not only ErbB1 but many other stimululi signaling as well.

Concluding remarks. Our results suggest that activation of ErbB1 by endogenous ligands, very likely EGF secreted from submandibular salivary glands, is involved in heart protection when male mice are exposed to an aggressive encounter. In addition, our results also suggest that exogenous intervention to activate ErbB1 may protect the heart against catecholamine-induced injury. These results, together with the recent observation that EGF prevents catecholamine-induced functional disturbances in perfused hearts and whole animals (34), suggest that it will be worth exploring the usefulness of external activation of ErbB1 receptor with EGF or other ligands of the EGF family to prevent reperfusion-induced injury after transient ischemia in which local release of catecholamines is involved (21). Indeed, further experiments in perfused hearts and isolated cardiomyocytes are required to demonstrate that activation of ErbB1 directly protects the heart.

DISCLOSURES

This study was supported by Dirección General de Enseñanza Superior e Investigación Científica Grant PB97-0936 from Ministerio de Educación y Ciencia, Spain.

REFERENCES


