Proteasome inhibition 1 h following ischemia protects GRK2 and prevents malignant ventricular tachyarrhythmias and SCD in a model of myocardial infarction

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Huang S, Patterson E, Yu X, Garrett MW, De Aos I, Kem DC. Proteasome inhibition 1 h following ischemia protects GRK2 and prevents malignant ventricular tachyarrhythmias and SCD in a model of myocardial infarction. Am J Physiol Heart Circ Physiol 294: H1298–H1303, 2008. First published January 11, 2008; doi:10.1152/ajpheart.00765.2007.—Arrhythmia-prone epicardial border zone (EBZ) tissue demonstrates decreased G protein-coupled receptor kinase-2 (GRK2) activity and increased sensitivity to isoproterenol 6–24 h after coronary artery ligation in the dog. We previously demonstrated that the ischemia-mediated decrease in GRK2 in cardiac ischemic tissue was largely blocked by proteasome blockade initiated 1 h before the onset of ischemia, and this was associated with significant cardioprotection against malignant ventricular tachyarrhythmias. For application to clinical circumstances, it is desirable to determine whether a clinical window exists following the onset of ischemia for such a protective effect. The treatment of six dogs with the selective proteasome inhibitor bortezomib 1 h after the surgical induction of left coronary artery ischemia provided 80% (EBZ) and 42% (infarct) protection (by immunoblot) against the loss of GRK2 at 24 h. There was no significant increase of heat shock protein 70(72) in the EBZ of bortezomib-treated animals compared with control. There was a striking absence of rapid (>300 beats/min) and very rapid (>360 beats/min) ventricular triplets that is highly predictive of sudden cardiac death (SCD) during electrocardiogram monitoring of the first 24 h in the bortezomib-treated animals in contrast with nontreated infarcted animals. There were no SCDs in the 6 treated animals (0%) and five SCDs in the 14 control animals (36%). Assay of whole blood proteasome activity demonstrated the expected decrease over the 24-h observation period. These data support the concept that proteasome inhibition within a window of time following myocardial infarction may be of use in suppressing malignant tachyarrhythmias and SCD.

β-adrenergic receptor; G-protein receptor kinase; sudden cardiac death

SUDDEN CARDIAC DEATH (SCD) is related to recurrent ventricular tachyarrhythmias arising from a thin layer of surviving myocardium adjacent to the infarcted tissue (4, 11, 16, 17). Two peaks of SCD are observed in dogs; one is within 30 min and the second is during a period from 6–24 h following the production of a myocardial infarction by stepped coronary artery ligation (CAL). In the later period, SCD is generally characterized by episodic tachyarrhythmias triggering electrophysiological events, including sustained ventricular tachycardia (VT) and ventricular fibrillation (7, 13, 15–17). In the dog, these late tachyarrhythmias represent primarily sustained reentrant tachycardia from the epicardial border zone (EBZ) tissue (15).

We have reported a marked decrease in total G protein-coupled receptor kinase-2 (GRK2) activity in whole slices of canine EBZ tissue obtained during the subacute 6–24 h period after infarction, resulting in a loss of the ability of the EBZ tissue to become desensitized to β-adrenergic stimulation (23, 24). Subsequent studies demonstrated that pretreatment of the dog by either of two agents, a proteasome inhibitor (22) or a TNF-α sequestrant (23), protected against the loss of GRK2 expression and significantly diminished the ventricular ectopic activity. Although no deaths from SCD occurred in the two treated groups compared with the control animals, the relatively small n for each prevented the reduction in SCD from reaching significance in the separate studies.

In the present study, we have examined the effect of administering the proteasome inhibitor bortezomib 1 h after an induction of a myocardial infarction to determine whether a potential therapeutic window exists. In addition, we have examined the effect of bortezomib on the induction of heat shock protein 70(72) [Hsp70(72)], since this induction has previously been claimed to have pro- and antiapoptotic activity. One proposed antiapoptotic pathway was through the Hsp70(72)-mediated suppression of certain mitogen-activated protein kinases (MAPKs) (5, 6, 12). In contrast, the bortezomib-protected kinase GRK2 is presumably effective because it is not destroyed by the proteasome pathway and remains active in contradistinction to the previously reported effect of Hsp70(72) on the MAPKs. For this reason, we wished to determine whether, in fact, Hsp70(72) was induced in the EBZ at a time when GRK2 was exerting its protective effect.

These data indicate that 1) a window following CAL-induced ischemia does exist for protection against the development of monomorphic ventricular tachyarrhythmias and SCD with bortezomib and 2) Hsp70(72) is not induced in the EBZ tissue at a time when GRK2 activity retains its expected beneficial effect in minimizing the impact of catecholamines on electrophysiological activity in the ventricle.

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 MATERIALS AND METHODS

Ischemic dog model. Twenty (14 control and 6 bortezomib treated) male dogs (30 to 45 lb) had a two-stage ligation of the left anterior descending coronary artery (LAD) below the first branch, as previously described (7, 8), during intravenous pentobarbital sodium (30 mg/kg) anesthesia. The minimal number of bortezomib-treated animals (n = 6) required to demonstrate significance had been experimentally determined by a previous study (22) and approved through overview by the Institutional Animal Care and Use Committee. Provision for an additional two animals was made in case circumstances such as an inadequate LAD system were present, but these extra animals were not needed. The last consecutively studied 14 control animals were used, including 6 new animals randomly intermixed with the study animals. In brief, a two-stage ligation of the coronary artery (a stenosis formed by ligating against and then removing a 20-gauge hypodermic needle, followed 15 min later by complete ligation of the artery) prevents or suppresses the initial lethal ventricular arrhythmias observed following abrupt coronary artery occlusion (7, 8). The dogs were given 0.4 mg/kg nalbuphine for postoperative analgesia and then allowed to awaken from anesthesia. A 24-h ambulatory ECG was obtained from leads on the thoracic surface. At 24 h after CAL, dogs were reanesthetized, a thoracotomy was performed, and the heart was removed and immersed in and perfused with ice-cold Tyrode buffer via the left main coronary artery. The outer 1.0 mm of EBZ tissue overlying the infarct was shaved by scalpel dissection. A cross section of the left ventricle was stained with triphenyltetrazolium chloride (TTZ; 0.1%) to determine the degree of viable and nonviable tissue. Remote site (RS) control tissue was obtained from a superior portion of the lateral left ventricle perfused via the left circumflex coronary artery. The infarct (Inf) sample was obtained from the middle of the region and characterized by darkened, reddish myocardium. The endocardial surface was also shaved, but owing to the thin nature of this tissue, it contained a significant amount of Inf tissue (22). The tissues were rapidly (within 10 min) divided into small aliquots and stored in liquid N2. The animal protocols were approved by the Institutional Animal Care and Use Committee and conform to the Helsinki International Guidelines for animal experimentation.

Bortezomib treatment. Six dogs were injected through a forepaw vein with 0.0875 mg/kg bortezomib (Velcade; Millennium Pharmaceuticals, Cambridge, MA). The other 14 dogs were injected with saline (control). The intravenous injection was given 1 h after LAD ligation. This dosage for dogs was based on previous studies of our laboratory and proteasome activity measurements (22). There are limited data available for dogs, but these studies (unpublished data; Millennium Pharmaceuticals) suggest a similar proteasome activity to that seen in our studies of this pathway might alter the frequency of malignant ventricular arrhythmias and the loss of GRK2 in this animal model. These animals were given bortezomib 0.0875 mg/kg iv 1 h after ligation of the LAD coronary artery. The effects of this infusion on whole blood cell proteasome activity (expressed as a chymotrypsin-to-trypsin activity ratio) are seen in Fig. 1. The proteasome activity in the whole blood of the dog was maximally suppressed at 1 h after infusion (2 h after LAD ligation) and gradually recovered to 50% of control at 24 h after ligation.

RESULTS

Each dog, following the two-stage ligation of the left anterior coronary artery, developed distinct Q waves on the anterior ECG leads. The cardiac tissue demonstrated a 1.0–1.5-mm surviving layer of myocardium on the epicardial surfaces of the Inf and a much thinner (0.2–0.5 mm) layer on the endocardial surface, as previously described (22). This was confirmed in each case by using a TTZ vital stain of a strip of tissue taken through the center of the infarct. Histological examination of the control RS, ischemic EBZ, and Inf tissues confirmed the expected changes 24 h after a transmural Inf.

The proteasome pathway has been implicated in the posttranscriptional degradation of GRK2 (18). We used the specific proteasome inhibitor bortezomib to determine whether blockade of this pathway might alter the frequency of malignant ventricular arrhythmias and the loss of GRK2 in this animal model. These animals were given bortezomib 0.0875 mg/kg iv 1 h after ligation of the LAD coronary artery. The effects of this infusion on whole blood cell proteasome activity (expressed as a chymotrypsin-to-trypsin activity ratio) are seen in Fig. 1. The proteasome activity in the whole blood of the dog was maximally suppressed at 1 h after infusion (2 h after LAD ligation) and gradually recovered to 50% of control at 24 h after ligation.

As previously reported, GRK2 expression after 24 h measured by immunoblot is significantly suppressed in both EBZ and Inf tissue taken from the saline control Inf animals (23, 24). Figure 2A demonstrates typical 24-h GRK2 expression in the various cardiac tissues in two dogs given either saline or bortezomib, respectively, 1 h after LAD ligation. Immunoblot analysis of unprecipitated GAPDH levels demonstrates equal amounts of the extracts. The cumulative data from cardiac tissues of six dogs are shown in Fig. 2B. Bortezomib treatment
conferred remarkable protection against the expected loss in GRK2 expression in the EBZ tissue with the mean of levels in this region 80% of their nonischemic RS tissue. There was also a significant protection for GRK2 in the Inf tissue up to 42% of the RS. Saline-treated ligated animals demonstrated a significant decrease of GRK2 in the EBZ and Inf tissue compared with the RS control tissue.

Hsp70(72) values were estimated by immunoblot to examine the possibility of a relationship with the protection of GRK2. As a loading control, samples were probed with anti-GAPDH antibodies. As shown in Fig. 3, there was no significant difference of Hsp70(72) between the RS, EBZ, and Inf tissues of the control and bortezomib-treated animals (RS, treated vs. untreated, $P = 0.27$; EBZ, treated vs. untreated, $P = 0.58$; and Inf, treated vs. untreated, $P = 0.37$; treated, $n = 6$; untreated, $n = 2$).

Holter ECG monitoring of the bortezomib-treated animals demonstrated a virtually complete absence of the very rapid triplets ($\geq 360$ beats/min) whose frequency is directly correlated with the occurrence of SCD in this animal model (Fig. 4) (15–17). This observation is in marked contrast to that observed in all survivors of the saline-treated (control) Inf animals ($P < 0.05$) and especially in those dying from SCD. This observation of a marked beneficial effect even extended to those rapid triplets with a somewhat slower rate of $\geq 300$ beats/min (Fig. 5). When the analysis was extended to the most rapid triplet observed within a given hour (Fig. 6), there again was a marked diminution ($P < 0.05$) in this analysis. No
bortezomib-treated animals died, whereas 5 of 14 control Inf animals died within the 24-h observation period. These data approached significance (bortezomib, 0 deaths of 6 dogs studied; and control, 5 deaths of 14 dogs studied; \( P = 0.06 \)). When the data from our previously published study using the six animals pretreated 1 h before the induction of ischemia (22) and those treated 1 h after ligation are combined and compared with the 14 consecutive saline control animals, this difference reaches significance (combined deaths for the bortezomib, 0 deaths of 12 dogs studied; and control, 5 deaths of 14 dogs studied; \( P < 0.05 \)).

**DISCUSSION**

The EBZ tissue represents an important site for the genesis of fatal sustained VT and SCD during the 5–24-h period following CAL in the dog (7, 13, 15–17). We have previously reported a rapid and consistent decrease in GRK2 expression and activity in the EBZ tissue overlying the myocardial Inf in a canine model (23, 24). This decrease was evident by 6 h after ligation of the LAD and persisted up to 96 h. These changes were associated with an increase in percentage of high-affinity \( \beta \)-adrenergic receptors (\( \beta \)-ARs) in the EBZ tissue and a loss in the ability of this tissue to desensitize normally to \( \beta \)-agonist stimulation of adenylyl cyclase activity. These observations help clarify the pathophysiology of this model of SCD and have major clinical implications, since they provide a rationale for the increased propensity of this tissue for \( \beta \)-AR agonist-stimulated proarrhythmic early and delayed afterdepolarizations (4, 11, 16, 17). GRK2 serves as one of several mechanisms for inhibiting maximal and/or sustained \( \beta \)-AR signal transduction (2, 3, 9, 19). A marked deficiency of GRK2 as observed in the EBZ tissues might be expected to exaggerate the risk for an electrophysiological myocardial event, and our previous studies are consistent with this understanding. Such an acquired tissue hypersensitivity to adrenergic agonists appears to be one of several potential biochemical mechanisms placing myocardial tissue at risk for such events. The loss of a \( \beta \)-AR modulator would support the clinical effectiveness of \( \beta \)-blockers when these pharmaceutical agents are used for first-line prevention of fatal cardiac arrhythmias (21). We have previously demonstrated that acute pretreatment of this animal model with the \( \beta \)-blocker nadolol markedly suppresses the malignant tachyarrhythmias and has a beneficial effect in preventing SCD (14, 16).

The effects of the proteasome inhibitor bortezomib on both ventricular tachyarrhythmias and the tissue expression of GRK2 were dramatic even when given 1 h after the induction of ischemia. There was a marked preservation of GRK2 in both the ischemic and infarcted tissues in the six animals. These data provide evidence that a window exists for the protection against GRK2 loss after an induction of ischemia. Suppression of whole blood proteasome activity appeared to be consistent in this animal model. Although there is compartmentalization of bortezomib in the body with less movement into the central nervous system, eyes, and testes (1), the whole blood concentration appears to reflect those in the heart, liver, and vascular tissues (unpublished data; Millennium Pharmaceuticals). The preservation of GRK2 expression in the Inf was only slightly less than that previously observed in animals given bortezomib during the preligation (\(-1\) h) period. These data support the limited, early access of collateral blood flow to this central region, which eventually becomes the Inf as seen at 24 h after ligation.

The effect of bortezomib in preserving GRK2 in the EBZ again was accompanied by a dramatic decrease in the very rapid (>360 beats/min) and rapid (>300 beats/min) ventricular triplets observed during Holter monitoring. The GRK2 protective effect of proteasome inhibition was of greater magnitude than that observed when a similar group of dogs was pretreated with a TNF-\( \alpha \) sequestrant etanercept (Enbrel; Amgen, Thousand Oaks, CA) (23). This marked preservation of GRK2 expression is concurrent with the dramatic protection against malignant triplets, which are highly associated with the onset of fatal tachyarrhythmias in this model. These data support the probability that GRK2 translocation to endosomal structures

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**Fig. 5.** The incidence (means ± SE) for all of the rapid triplets (all ≥300 beats/min) for the bortezomib and saline control groups. The saline control animals had a significantly greater number of total triplets at the indicated time points (hours on the x-axis) (\( P < 0.05 \)). There was no significant difference between the bortezomib-pretreated animals and those given bortezomib 1 h after LAD ligation. \( n \), Number of dogs. *Times of death of the 5 dogs in the control group.

**Fig. 6.** The mean intrinsic rate (beats/min) for the fastest ventricular triplet observed in each dog during each hour for the 4- to 24-h period following LAD ligation in control (\( n = 14 \) dogs) and bortezomib-pretreated (\( n = 6 \) dogs) animals and those treated 1 h after LAD ligation (\( n = 6 \) dogs). The saline control group consistently had faster triplets compared with the bortezomib groups at the indicated time points (hours on the x-axis) (\( P < 0.05 \)), which would put them at greater risk for developing monomorphic ventricular tachycardia and subsequent ventricular fibrillation.
and/or a marked activation of the proteasome proteolytic pathway (18) may combine to account for the marked diminution of GRK2 protein expression in this animal model of human SCD and lead to increased sensitivity of the tissues to autonomic activity.

Another highly selective proteasome inhibitor PS-519 has been demonstrated to reduce reperfusion injury when given to a pig model (20). The Pye et al. (20) study, however, was directed toward an entirely different set of cellular functions, resulting in the reperfusion activation of apoptosis and cellular injury. This benefit was attributed to the suppression of NF-κB activation and reduced myocardial inflammation and subsequent reperfusion injury. Others have noted that proteasome inhibition leads to a variable effect on cardiomyocytes and to other cell lines. Interestingly, there are reports of proteasome inhibition enhancing cell death by increasing MAPK JNK and p38 activity and, at the same time, inhibiting this same activity by an induction of Hsp70(72). This leaves the cells to respond to opposing effects that are beneficial or detrimental to protection against apoptosis (12).

Since their report of suppression of kinase activity was just the opposite of our extensive data regarding GRK2, we looked for concordant or discordant changes in Hsp70(72) in our model for SCD. We found no significant correlation of changes in Hsp70(72) between bortezomib-treated and -untreated animals. Since these data were obtained at 24 h, it is possible that the induction of Hsp70(72) might have occurred during the early hours after LAD ligation. Since the GRK2 was maintained over the full 24 h and ischemia persisted, it is likely that any early changes in Hsp70(72) that would be protective and were not sustained would have been associated with a loss of GRK2. Since the proteasome pathway has already been associated directly with GRK2 degradation (2, 18), it is unlikely that Hsp70(72) has any significant effect on the protection of GRK2 or its activity.

The present studies continue to support the concept that acute, selective inhibition of the 20S proteasome may be a useful tool for examining pathophysiological sequelae of myocardial ischemia. Although the association of GRK2 preservation with antecedent proteasome inhibition was of interest, the present studies now directly support the concept that bortezomib or another inhibitor of proteasome activity may protect against the onset of malignant tachyarrhythmias and consequent SCD.

In summary, our data using in vivo infarcted hearts has repetitively shown a rapid and marked decrease of GRK2 activity and protein expression in the EBZ and subendocardial tissues adjacent to the Inf. Preservation of GRK2 is observed in both EBZ and Inf tissue when proteasome activity is inhibited before or 1 h after the induction of ischemia. This suggests that protection of GRK2 expression and activity in the myocardium may provide an alternative or complementary means for decreasing the occurrence of postischemic SCD.

REFERENCES


