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Blockade of Electron Transport During Ischemia Preserves bcl-2 and Inhibits Opening of the Mitochondrial Permeability Transition Pore

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Abstract

Myocardial ischemia damages the electron transport chain and augments cardiomyocyte death during reperfusion. To understand the relationship between ischemic mitochondrial damage and mitochondrial-driven cell death, the isolated perfused heart underwent global stop-flow ischemia with and without mitochondrial protection by reversible blockade of electron transport. Ischemic damage to electron transport depleted bcl-2 content and favored mitochondrial permeability transition (MPT). Reversible blockade of electron transport preserved bcl-2 content and attenuated calcium-stimulated mitochondrial swelling. Thus, the damaged electron transport chain leads to bcl-2 depletion and MPT opening. Chemical inhibition of bcl-2 with HA14-1 also dramatically increased mitochondrial swelling, augmented by exogenous H2O2 stress, indicating that bcl-2 depleted mitochondria are poised to undergo MPT during the enhanced oxidative stress of reperfusion.

1.0 Introduction

Mitochondrial dysfunction contributes to myocardial injury during ischemia-reperfusion [1]. Ischemia results in damage to the electron transport chain (ETC) and decreased rates of oxidative phosphorylation [2,3]. Reperfusion after ischemia does not result in additional damage to electron transport [4,5], although, in contrast to mitochondria, substantial cardiomyocyte injury occurs during reperfusion [6-8]. Mitochondrial-dependent cardiac injury involves the increased production of reactive oxygen species (ROS) [9-12], the depletion of anti-apoptotic proteins from mitochondria [13,14], and increased susceptibility to opening of the mitochondrial permeability transition pore (MPT) [14-17]. Protection of mitochondria against ischemic damage to the ETC by the reversible blockade of electron transport during ischemia [18,19] or other pharmacological treatments [20-22] decreases

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myocardial injury assessed following reperfusion [4,23,24], thus establishing a link between damage to electron transport during ischemia and cardiomyocyte death during reperfusion.

Although decreased activity of the electron transport chain could contribute to myocardial injury during reperfusion via decreased respiration and energy production, reperfused myocardium can be protected by intervention only during reperfusion. Brief, reversible blockade of electron transport during reperfusion [23,25] or the use of postconditioning consisting of brief periods of intermittent ischemia [26], protect reperfused myocardium despite the persistence of ischemia-induced ETC damage during reperfusion [26,27]. Thus, mitochondrial-dependent processes other than decreased oxidative phosphorylation must account for the mitochondrial-dependent injury observed during reperfusion. The ETC-dependent processes that generate cardiac injury during reperfusion remain unclear.

The mitochondrial permeability transition pore (MPT) is a non-selective pore spanning the inner and outer mitochondrial membranes. MPT opening is a key contributor to cardiac injury during ischemia-reperfusion [28]. MPT opening is favored at the onset of reperfusion due to increased oxidative stress, rapid normalization of intracellular pH, and mitochondrial calcium loading. [15,16,28,29]. Ischemic damage to the electron transport chain increases ROS generation during re-oxygenation [10,30], whereas prevention of ischemic damage decreases ROS generation during reperfusion [4,31]. Thus, ischemic damage to the ETC may contribute to cardiac injury during reperfusion via ROS generation that facilitates MPT opening. The permeability of the outer mitochondrial membrane is also regulated by the expression of bcl-2 family proteins [13,32]. A decreased content of anti-apoptotic proteins (bcl-2, bcl-xl) and/or the increased content of pro-apoptotic proteins (bax and bak) will lead to permeation of the outer membrane and cytochrome *c* loss [13,32]. Ischemia-reperfusion decreases myocardial bcl-2 content in the isolated heart [33] and bcl-2 inhibition with the small molecule HA14-1 abrogates cardioprotection [34]. However, the potential electron transport chain dependence of bcl-2 depletion is unknown.

Blockade of the proximal electron transport chain protects mitochondria during ischemia [19], providing an experimental model to identify and study the mechanisms of ETC-dependent cardiac injury. Mitochondria were studied at the end of ischemia, in order to exclude potential contributions of in situ reperfusion to mitochondrial damage. The current study found that bcl-2 depletion from mitochondria during ischemia is indeed ETC dependent. Decreased bcl-2 content, perhaps in concert with increased ROS generation from the damaged ETC, increases the probability of mitochondrial permeability transition. Thus, an increased predisposition to permeability transition and activation of programmed cell death are complimentary, reinforcing mechanisms that translate ETC damage from ischemia into cardiomyocyte death during reperfusion.

2.0 Methods

2.1 Isolated rabbit heart model of ischemia and reperfusion

The Animal Care and Use Committees of the Louis Stokes VA Medical Center and Case Western Reserve University approved the protocol. The isolated rabbit heart perfusion protocol was performed as described previously [3,5] (Supplemental Methods). Untreated ischemic hearts were first perfused with Krebs-Henseleit buffer for 15 min. followed by 30 min. stop-flow ischemia. In amobarbital treated ischemic hearts, amobarbital (2.5 mM) [18] in oxygenated Krebs-Henseleit buffer was infused for 1 min. immediately before ischemia. Time control hearts were perfused for 45 min. without ischemia [2].

There were no differences in hemodynamic parameters between time control, untreated ischemia, and amobarbital treated ischemia groups at the end of the 15 min. equilibration

period before the infusion of amobarbital (Supplemental Table 1). Developed pressure was maintained during 45 min perfusion in time control hearts (88±6 at 15 min equilibration and 85±1 mmHg at end of 45 min perfusion). Ischemia led to myocardial contracture and markedly increased diastolic pressure compared to the pre-ischemic value. Amobarbital treatment significantly attenuated the increase in diastolic pressure compared to the untreated heart as previously described (Supplemental Table 1).

2.2 Isolation and analysis of two populations of cardiac mitochondria

At the end of ischemia, hearts were removed from the perfusion column and placed into Chappel-Perry buffer [(in mM) 100 KCl, 50 MOPS, 1 EGTA, 5 $MgSO_4$•7 H_2O, and 1 ATP; pH 7.4] at 4°C. Cardiac mitochondria were isolated according to Palmer et al. [35], with minor modifications as previously described [3] (Supplemental Methods). Oxygen consumption in mitochondria was measured using a Clark-type oxygen electrode at 30°C (Stratkelvin, Scotland, UK) in a solution containing 80 mM KCl, 50 mM MOPS, 1 mM EGTA, 5 mM KH_2PO_4, and 1 mg/ml BSA, at pH 7.4. Glutamate (complex I substrate), and TMPD-ascorbate plus rotenone (complex IV substrate) were used as electron donors [3]. Cytochrome contents were measured in mitochondria solubilized in 2% deoxycholate in 10 mM sodium phosphate buffer using the difference of sodium dithionite reduced and air oxidized spectra [3,36]. H_2O_2 production from intact mitochondria was measured using the oxidation of the fluorogenic indicator amplex red in the presence of horseradish peroxidase (HRP) [37]. Glutamate (10 mM) and succinate (10 mM) were used as substrates.

2.3 Western blot analysis

SSM were solubilized and boiled for 5 min in buffer including 4% (w/v) SDS, 1 mM 2-mercaptoethanol, 10 mM Tris/HCl (pH 6.8) and 10% (w/v) glycerol. Equal amounts of protein (100 μg) were loaded onto 14% SDS-PAGE, run and transferred to PVDF membrane. The membranes were first blotted by 5% non-fat milk for one hour. Then membranes were blotted with primary antibodies for one hour or overnight. Antibodies to bcl-2 (catalog number: 2876) bax (2722), and subunit 4 of cytochrome oxidase (4844) were purchased from Cell Signaling Technology (Danvers, MA). The blots were incubated with peroxidase conjugated anti-rabbit antibody (1:5000) for 1 hour prior to ECL detection. The intensities of blotting were quantified by densitometer (GS800 densitometer, Golden Valley, MN).

2.4 Mitochondrial swelling analysis

Opening of the mitochondrial pore was determined by Ca^{2+} induced swelling of isolated mitochondria measured as a reduction in A_{520}. Isolated SSM or IFM (0.25 mg/ml) were resuspended in swelling buffer (120 mM KCl, 5 mM KH_2PO_4, 50 mM MOPS, pH 7.4) with succinate (10 mM) as substrate. Absorbance was determined at 520 nm and MPT induced by 200 uM $CaCl_2$. Cyclosporine A (1 μM) inhibited swelling by approximately 90% (Figure 4), confirming the decrease in absorbance was due to MPT. Results were expressed as Δ change of absorbance /mg protein [38]. To test the effect of HA14-1 on MPT opening, isolated control rabbit SSM were incubated with HA14-1 (20 uM) [39] in the absence and presence of H_2O_2 (100 uM) [40] for 5 min. at 30 °C and mitochondrial swelling was determined.

2.5 Statistical Analysis

Data are expressed as the mean ± standard error of the mean. Not every sample was available for each assay and led to different animal numbers in different assays. Differences among groups were compared by one-way analysis of variance with post hoc comparisons performed using the Student-Newman-Keuls test of multiple comparisons. A difference of

$p<0.05$ was considered significant (SigmaStat for Windows Version 1.0, Jandel Corporation).

3.0 Results

3.1 Blockade of electron transport protects oxidative phosphorylation in SSM during ischemia

Thirty minutes of ischemia decreased the maximal rate of ADP-stimulated respiration in SSM and IFM with glutamate as substrate, as previously described in this model [2,3]. In contrast, glutamate oxidation in SSM isolated from hearts with blockade of electron transport during ischemia due to amobarbital-treatment was similar to rates observed in SSM from time control hearts (Table 1). Ischemia also decreased dinitrophenol-uncoupled respiration in SSM and IFM compared to time control, and this defect was prevented by amobarbital treatment (Table 1). Oxidative phosphorylation through cytochrome oxidase also decreased in SSM following ischemia. Amobarbital treatment preserved respiration through cytochrome oxidase, indicating that blockade of the proximal electron transport chain protects the distal electron transport chain against ischemic damage (Table 1). Ischemia did not damage the distal electron transport chain in IFM (Table 1), consistent with previous findings [2,3].

3.2 Blockade of electron transport during ischemia prevents bcl-2 depletion

Ischemia decreased the content of bcl-2 in SSM compared to time control, whereas blockade of electron transport during ischemia preserved bcl-2 content (Figure 1). In contrast, ischemia did not substantially alter the bax content in SSM compared to time control or amobarbital treated SSM (Figure 1). Equal protein loading was confirmed by the detection of subunit IV of cytochrome oxidase (Figure 1). These results indicate that the ETC lies upstream of and contributes to bcl-2 depletion during ischemia. Bcl-2 could not be detected in IFM, likely secondary to the protease treatment used during isolation.

3.3 Blockade of electron transport during ischemia decreases net H_2O_2 production from SSM

Ischemic damage to the ETC increased the net production of H_2O_2 in SSM oxidizing glutamate as a complex I substrate. The increased H_2O_2 generation from SSM is also observed following ischemia with succinate as a complex II substrate in the presence of rotenone. Amobarbital treatment during ischemia decreased ROS generation from the ETC (Figure 2). Thus, ischemic damage to electron transport increased the net ROS production. Ischemia also tended to increase H_2O_2 generation from IFM isolated after ischemia when glutamate is the substrate, but the results did not reach the statistical difference (Figure 2).

3.4 Blockade of electron transport during ischemia preserves cytochrome c content

The cytochrome *c* content in SSM decreased after 30 minutes of ischemia, whereas amobarbital treatment preserved cytochrome *c* content (Supplemental Table 2). Other cytochrome contents (c_1, *b*, aa_3) were unaffected. Ischemia did not alter cytochrome contents in IFM (Supplemental Table 2).

3.5 Blockade of electron transport during ischemia attenuates the opening of MPT

Ischemia significantly increased the calcium-induced swelling of SSM compared to time control. The SSM protected by amobarbital treatment during ischemia exhibited substantially decreased swelling compared to the SSM isolated from untreated ischemic hearts (Figure 3). Ischemia also led to calcium-induced swelling in IFM, but the rate and extent of swelling was less than in SSM (Figure 3).

3.6 Inhibition of bcl-2 markedly induced MPT opening during oxidative stress

Mitochondrial swelling was used as an in vitro index of the susceptibility to opening of the permeability transition pore [38]. In the original tracing portion of Figure 4 (top), calcium (200 uM) stimulation led to mitochondrial swelling indicated by a decrease in absorbance at 520 nm (A_{520}). H_2O_2 stimulation or HA14-1 treatment increased swelling in control SSM (Figure 4 top and bottom). In addition, H_2O_2 stimulation in the presence of HA14-1 dramatically increased swelling compared to H_2O_2 or HA14-1 treatment alone. Cyclosporine A prevented H_2O_2 or HA14-1 stimulated swelling, indicating that swelling occurred via MPT opening (Figure 4 top and bottom).

4.0 Discussion

Blockade of electron transport during ischemia decreases ROS production and the probability of MPT opening. Ischemia decreases bcl-2 content whereas protection of the electron transport chain by amobarbital inhibition preserves the bcl-2 content, providing strong support that the electron transport chain contributes to the previously observed [41,42] bcl-2 depletion during ischemia. The decreased bcl-2 content facilitates MPT opening in the presence of oxidative stress. The current results indicate that the ischemia-induced decrease in bcl-2 content in combination with increased ROS generation from the damaged electron transport chain will trigger MPT opening and increase cardiac injury during early reperfusion.

Mitochondria are the key mediators of cardiomyocyte survival and death during ischemiareperfusion, mediated in large part via MPT opening [28,32]. The exact components of the MPT are not clear, although cyclophilin D in the mitochondrial matrix regulates MPT opening [43]. Although MPT may open during cardiac ischemia [44-46], MPT opening mostly occurs during reperfusion [14,15,17,28,32,47-49]. Electron microscopy discloses that mitochondria remain intact in the isolated rabbit heart at the end of ischemia [3], supporting that MPT does not open during ischemia *in situ* [17]. The burst of ROS generation and rapid normalization of intracellular pH during early reperfusion are key factors that induce MPT [28,29]. Ischemic damage to electron transport increases ROS generation in isolated mitochondria [10]. In contrast, reversible blockade of electron transport only during ischemia decreases ROS generation from mitochondria isolated following reperfusion [4]. Moreover, reversible blockade of electron transport decreases in situ ROS generation during reperfusion [31]. Thus, the ETC is the source of ROS generation, and ischemia-damaged mitochondria release ROS during reperfusion [11]. In the present study, we found that the ischemia-damaged electron transport chain increased ROS generation accompanied by mitochondrial swelling. In isolated mitochondria, oxidative stress induced by exogenous H_2O_2 increased mitochondrial swelling. These results suggest that the ischemia-damaged ETC increases MPT opening by inducing ROS generation.

Although SSM were isolated at the end of ischemia, the swelling assay was conducted under the conditions of physiological pH, oxygen content, substrate, and high concentration of calcium. This condition is similar to the intracellular environment encountered by mitochondria during early reperfusion. In the same milieu, SSM isolated from ischemic hearts yet without ischemic damage as a result of amobarbital treatment did not exhibit significant swelling, indicating that damage to the ETC from ischemia was required for MPT opening. Our results support that ischemic damage to the ETC is a mechanism of MPT opening during reperfusion.

Bcl-2 is an anti-apoptotic protein located on the outer mitochondrial membrane and endoplasmic reticulum [13,50]. Over-expression of bcl-2 decreases ischemia-reperfusion injury in isolated hearts [42], whereas functional inhibition of bcl-2 with HA14-1, a small

molecule that prevents bcl-2 inhibition of pro-apoptotic proteins [40], blocked cardioprotection [34]. Inhibition of bcl-2 with HA14-1 stimulates mitochondrial swelling [34], indicating that manipulation of bcl-2 function impacts MPT opening [13,14]. In the current study, ischemic damage to the ETC decreased bcl-2 content and favored MPT opening, whereas blockade of electron transport during ischemia prevented the loss of bcl-2 in association with the decreased mitochondrial swelling. In isolated mitochondria, inhibition of bcl-2 increased calcium-stimulated mitochondrial swelling, and swelling was further enhanced in the presence of oxidative stress. At the onset of reperfusion, re-oxygenation leads to ROS generation from the ischemia-damaged ETC [4,31,51]. This oxidative stress occurs in the setting of decreased mitochondrial bcl-2 content and favors MPT opening, eventually leading to the ischemia-reperfusion injury [28]. Thus, preservation of bcl-2 content via reversible blockade of electron transport contributes to reduced myocardial injury during ischemia-reperfusion by inhibition of MPT opening.

Damage to the ETC occurs mainly during ischemia [3,27,52] and persists during reperfusion [5,26]. The unexpected finding that interventions that protect myocardium during reperfusion, including postconditioning [26,47] and brief inhibition of electron transport [23,25] are effective without the recovery of oxidative phosphorylation [26] point to ETC dependent processes other than respiration as key effectors of mitochondrial-driven cardiac injury. In the present study, enhanced net production of oxidants, shift of the mitochondrial outer membrane proteome to a pro-apoptotic ensemble via depletion of bcl-2, and enhanced susceptibility to mitochondrial permeability transition are mechanisms that transduce the electron transport chain damage caused by ischemia into cardiomyocyte death during reperfusion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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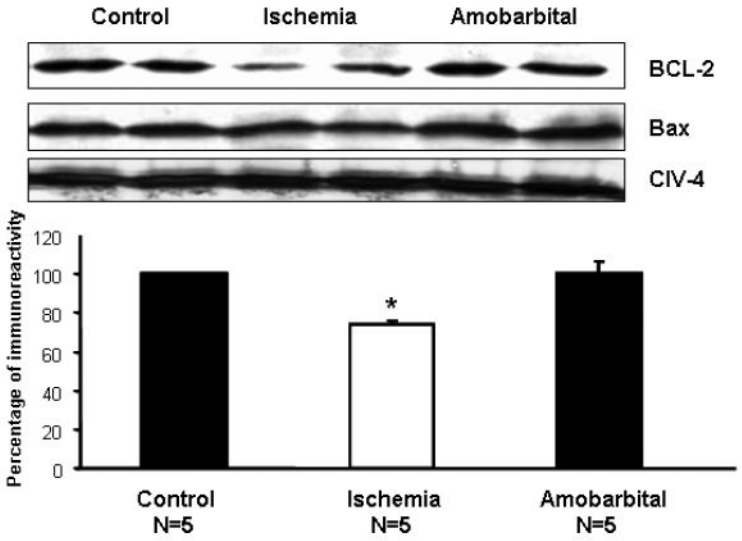

Figure 1. Blockade of electron transport during ischemia preserves bcl-2 content in SSM

Ischemia markedly decreases the bcl-2 content in SSM isolated from rabbit heart compared to time control, whereas amobarbital prevents bcl-2 depletion during ischemia. Ischemia did not alter bax content compared to time control or amobarbital treated hearts. The upper panel consists of representative immunoblots, and the lower panel is the quantitation using densitometery. (Mean±SEM; *$p<0.05$ vs. time control; † $p<0.05$ vs. untreated-ischemia).

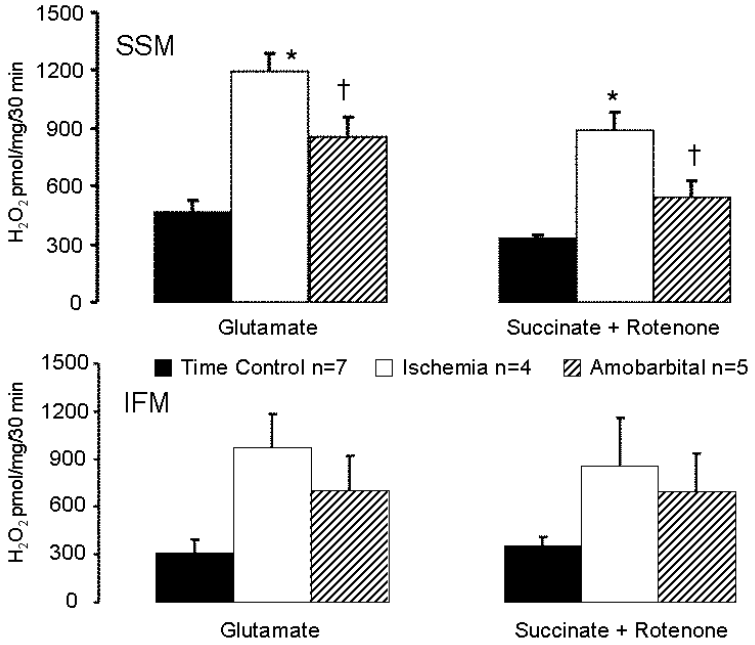

Figure 2. The net release of H_2O_2 from isolated mitochondria in the presence and absence of ischemia

Compared to SSM from time control hearts, ischemic damage to electron transport chain increases net H_2O_2 production from SSM using glutamate as complex I substrate and succinate+rotenone as a complex II substrate. In contrast, protection of SSM by blockade of electron transport during ischemia by amobarbital decreases the H_2O_2 generation compared to the untreated ischemia (upper panel). Ischemia also tends to increase H2O2 generation in IFM compared to time control (lower panel). (Mean±SEM; *$p<0.05$ vs. time control; † $p<0.05$ vs. untreated-ischemia).

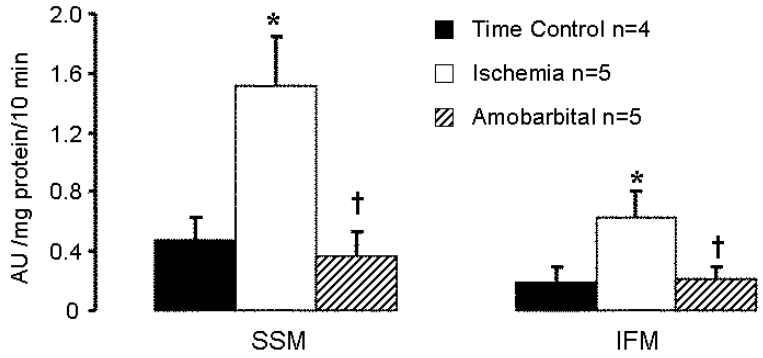

Figure 3. Blockade of electron transport during ischemia prevents calcium-induced swelling in SSM following ischemia

Ischemia leads to a significant increase in calcium-stimulated swelling in SSM. Blockade of electron transport protects SSM and limits the swelling compared to untreated mitochondria following ischemia. Ischemia also induces calcium stimulated swelling in IFM, albeit to a lesser degree. (Mean±SEM; *$p<0.05$ vs. time control; † $p<0.05$ vs. untreated ischemic group).

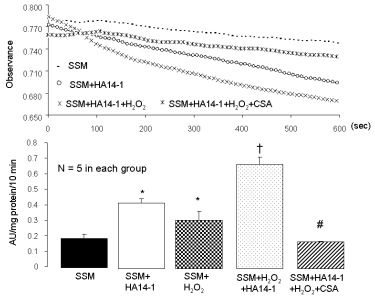

Figure 4. Inhibition of bcl-2 with the small molecule inhibitor HA14-1 increases mitochondrial swelling in SSM isolated from non-ischemic control rabbit hearts

In addition, there is an additive effect when H_2O_2 is applied in the presence of HA14-1. The upper panel shows representative tracings (to preserve clarity, only select tracings are shown). The lower panel shows the summed results of mitochondrial swelling induced by the bcl-2 inhibitor, H_2O_2 treatment, bcl-2 inhibitor + H_2O_2 treatment, and bcl-2 inhibitor + H_2O_2 + cyclosporine A (CSA). (Mean±SEM; *$p<0.05$ vs. control SSM; † $p<0.05$ vs. other groups; # $p<0.05$ vs HA14-1 + H_2O_2). HA14-1 and H_2O_2 stimulation results in CSA-sensitive mitochondrial swelling, indicting that these treatments increase MPT opening.

Table 1

Blockade of electron transport with amobarbital preserves respiration following ischemia.

	Time control (n=7)	Ischemia (n=9)	AMO (n=10)
	Subsarcolemmal Mitochondria (SSM)		
Glutamate-ADP	175±10 104±8*		167±8†
Glutamate-DNP	155±10	102±10*	157±11†
TMPD-asc-ADP	504±17	315±21*	540±31†
	Interfibillilar Mitochondria (IFM)		
Glutamate-ADP	252±10 186±18*		260±12†
Glutamate-DNP	252±20	189±27*	273±24†
TMPD-asc-ADP	858±53	714±22	810±49

	SSM		IFM	
	Glutamate	TMPD-ascorbate	Glutamate	TMPD-ascorbate
Time control (n=7)	175±10	504±17	252±10	858±53
Ischemia (n=9)	104±8*	315±21*	186±18*	714±22
AMO (n=10)	167±8†	540±31†	260±12†	810±49

Data are expressed as Mean ± SEM.

*P<0.05 vs. time control

†P<0.05 vs. ischemia. AMO, amobarbital + ischemia ADP, 2 mM; DNP (dinitrophenol, 0.3 mM).