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## Multiplicity of Effectors of the Cardioprotective Agent, Diazoxide

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### Abstract

Diazoxide has been identified over the past 50 years to have a number of physiological effects, including lowering the blood pressure and rectifying hypoglycemia. Today it is used clinically to treat these conditions. More recently, another important mode of action emerged: diazoxide has powerful protective properties against cardiac ischemia. The heart has intrinsic protective mechanisms against ischemia injury; one of which is ischemic preconditioning. Diazoxide mimics ischemic preconditioning. The purpose of this treatise is to review the literature in an attempt to identify the many effectors of diazoxide and discuss how they may contribute diazoxide's cardioprotective properties. Particular emphasis is placed on the concentration ranges in which diazoxide affects its different targets and how this compares with the concentrations commonly used to study cardioprotection. It is concluded that diazoxide may have several potential effectors that may potentially contribute to cardioprotection, including  $K_{ATP}$  channels in the pancreas, smooth muscle, endothelium, neurons and the mitochondrial inner membrane. Diazoxide may also affect other ion channels and ATPases and may directly regulate mitochondrial energetics. It is possible that the success of diazoxide lies in this promiscuity and that the compound acts to rebalance multiple physiological processes during cardiac ischemia.

### Keywords

Cardioprotection; Ischemia; Ischemic preconditioning; Diazoxide;  $K_{ATP}$  channels; Mitochondria

### Introduction

Diazoxide (CAS Number: 364-98-7; 7-chloro-3-methyl-2H-1,2,4-benzothiadiazine 1,1-dioxide; Figure 1) has a molecular weight of 230.7 and a molecular formula of  $C_8H_7ClN_2O_2S$ . It is a white powder insoluble in water, but soluble in organic solvents (e.g. 10 mg/ml in DMSO). The dogma has arisen in recent years (particularly in the cardioprotection literature) that diazoxide is an agent with a unique molecular target. This is not the case and the purpose of this literature review is to highlight the multiplicity of diazoxide effectors to assist in a better understanding of mechanisms involved in the established cardioprotective effects of this compound.

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### Conflict of Interest statement

The author declares that there are no conflicts of interest

## History

In the early 1960's, a study was designed to examine possible non-diuretic mechanisms by which benzothiadiazines lower blood pressure - diazoxide was found to directly cause vasodilation of blood vessels independent of diuretic actions (Rubin, et al., 1962). Early reports, however, also demonstrated that some hypotensive drugs such as diazoxide led to elevated blood glucose levels (hyperglycemia) (Okun, et al., 1964; F. Wolff, 1964). The following years saw a large rise in publications, mostly related to the hypotensive and hyperglycemic effects of diazoxide (Figure 1). Other actions, including effects on renal excretory function, also started to emerge (Johnson, 1971; Rubin, et al., 1968). Nevertheless, the compound became accepted for its oral use in the management of intractable hypoglycemia and intravenously in the management of hypertensive emergencies. The publication rate waned in the mid-1980's. Following the identification of diazoxide molecular effectors in pancreatic  $\beta$ -cells (Henquin & Meissner, 1982; Trube, et al., 1986) and vascular smooth muscle cells (Standen, et al., 1989), a secondary rise in diazoxide-related publications occurred (Figure 1), which was further stimulated by the mid-1990's findings that diazoxide has powerful cardioprotective properties (Garlid, et al., 1997; Nakai & Ichihara, 1994).

## Clinical use

In tablet form (e.g. Proglycem, FDA approved in 1976) diazoxide is prescribed orally (usually 2 to 3 times daily) for the management of symptomatic hypoglycemia. Side effects include shortness of breath, swelling in extremities, tachycardia, chest pain, blurred vision, bruising or bleeding, unusual weakness; and decreased frequency of urination. Intravenously (e.g. Hyperstat) diazoxide is indicated as a peripheral vasodilator for short-term use in the emergency reduction of blood pressure in severe, nonmalignant and malignant hypertension in hospitalized adults; and in acute severe hypertension in hospitalized children, when prompt and urgent decrease of diastolic pressure is required. Diazoxide is also used to treat hypoglycemia that results from congenital hyperinsulinism of infancy (HI) (Hussain, et al., 2004). The mechanisms of diazoxide's clinical action relate predominantly to opening of pancreatic and smooth muscle  $K_{ATP}$  channels, as will be discussed in subsequent sections.

## Diazoxide is cardioprotective against ischemic insults

During the treatment of patients with hypotension, early studies suggested an increase in myocardial injury with diazoxide (e.g. chest pain and ST elevation) (Kanada, et al., 1976; O'Brien, et al., 1975). These effects may have been related to the hypotensive action of the drug. Most controlled animal studies to date, however, as well as *in vitro* studies with human cardiac tissues, suggest that diazoxide has cardioprotective properties (Garlid, et al., 1997; Nakai & Ichihara, 1994; Y. Wang, et al., 1999). Intrinsic adaptive physiological processes within the myocardium render the heart more resistant to potentially lethal ischemic injury. One of the protective phenomena is ischemic preconditioning (IPC) - the most powerful means of delaying myocardial injury that has been identified to date (Yellon & Downey, 2003). Diazoxide is powerfully anti-ischemic and/or recapitulates the cardioprotective effects of IPC. A full review of these studies is beyond the scope of this review and some of these studies are highlighted in Table 1. The cardioprotective effects are observed in a variety of species (rat, rabbit, dog and human), with *ex vivo* and *in vivo* methods and over a concentration range of  $\sim 10$ – $100 \mu\text{M}$  (or 1–10 mg/kg intravenously). Most studies utilize a single dose of diazoxide. In one study, the  $\text{EC}_{25}$  for diazoxide's protective effect (measured as an increased the time to onset of contracture) in isolated rat hearts subjected to 30 min global ischemia was reported to be  $\sim 10 \mu\text{M}$  (Garlid, et al., 1997). The  $K_{1/2}$  is likely to be higher. In another study, the optimal protective concentration in isolated rat hearts subjected

to ischemia/reperfusion was reported to be 80  $\mu\text{M}$  (Y. Wang, et al., 1999). With instrumented dogs, 80  $\mu\text{M}$  (but not 8  $\mu\text{M}$ ) diazoxide was reported to provide partial protection against the development of a post-ischemic infarct (Sanada, et al., 2001). The protective effect of diazoxide is equivalent to that of ischemic preconditioning (IPC) and diazoxide is often used as a pharmacological means to induce preconditioning. Moreover, both the IPC- and diazoxide-induced protection is minimized by tolbutamide, HMR-1883 or glibenclamide (sulfonylurea compounds that block various types of  $\text{K}_{\text{ATP}}$  channels (Escande, 1989; Faivre & Findlay, 1989; Gogelein, et al., 1998; Quayle, et al., 1995; Trube, et al., 1986)) or 5HD (often used as a mitochondrial  $\text{K}_{\text{ATP}}$  blocker, but which also has other off-target effects; see later) (Birincioglu, et al., 1999), suggesting a causative link between the diazoxide effector(s) and mechanism(s) involved in IPC. The purpose of this mini-review is to highlight the multiplicity of diazoxide effectors.

### Diazoxide activates pancreatic $\beta$ -cell $\text{K}_{\text{ATP}}$ channels

Diazoxide has long been recognized to be hyperglycemic by inhibiting insulin release from pancreatic  $\beta$ -cells (Loubatieres, et al., 1966; Okun, et al., 1964; F. W. Wolff, et al., 1963). The mechanism was found to be an increased membrane  $\text{K}^+$  permeability (measured with  $\text{Rb}^+$  flux assays), leading to membrane hyperpolarization, inhibition of  $\text{Ca}^{2+}$  influx (Henquin & Meissner, 1982) and diminished insulin secretory release. Shortly after the discovery of cardiac  $\text{K}_{\text{ATP}}$  channels (Noma, 1983; Trube & Hescheler, 1984), similar channels in the pancreatic  $\beta$ -cell were found to be responsible for the diazoxide-induced increase of  $\text{K}^+$  permeability (Trube, et al., 1986). The diazoxide-sensitivity of the pancreatic  $\beta$ -cell  $\text{K}_{\text{ATP}}$  channel is high ( $\text{K}_{1/2}$  of 7–20  $\mu\text{M}$ ; Table 2), which is in the same concentration range as the drug's cardioprotective benefit (Table 1). Pancreatic  $\beta$ -cell  $\text{K}_{\text{ATP}}$  channels are unlikely to be involved in diazoxide's cardioprotective effects when using *ex vivo* preparations (e.g. isolated hearts). In patients, however, or when using diazoxide with *in vivo* experimental approaches, the possibility must be considered that diazoxide may elevate blood glucose levels, which in turn may influence the ischemic outcome. Provision of glucose, together with insulin and potassium (GIK), has clear beneficial effects during ischemia (Opie, 1975) but the benefit of increasing glucose levels alone is questionable (LaDisa, et al., 2004; Spath, et al., 1976).

### Diazoxide activates smooth muscle $\text{K}_{\text{ATP}}$ channels

The hypotensive properties of diazoxide have been described over 50 years ago (Rubin, et al., 1962). As is the case for other  $\text{K}_{\text{ATP}}$  channel openers (Gross, et al., 1989; Quayle, et al., 1997; S. Sakamoto, et al., 1989), the vasodilatory effects of diazoxide in vascular smooth muscle is due to opening of vascular  $\text{K}_{\text{ATP}}$  (or  $\text{K}_{\text{NDP}}$ ) channels (Standen, et al., 1989), which leads to local relaxation in smooth muscle due to an increased membrane  $\text{K}^+$  permeability, subsequent inhibition of excitability, lowered cytosolic  $\text{Ca}^{2+}$  and relaxation. Patch clamp data demonstrated that the diazoxide sensitivity of smooth muscle  $\text{K}_{\text{ATP}}/\text{K}_{\text{NDP}}$  channels is in the same concentration range as that of pancreatic  $\beta$ -cell  $\text{K}_{\text{ATP}}$  channels (Table 2).  $\text{K}_{\text{ATP}}$  channels have a pronounced role in controlling coronary blood flow and the coronary reserve, particularly in the resistance arterioles (Akai, et al., 1995; Sato, et al., 1994). At 2.5mg/kg (within the cardioprotective concentration range; Table 1) intravenous diazoxide causes a 180% increase in coronary flow in anesthetized dogs (Scott & Cowley, 1969). In some studies diazoxide was noted to improve coronary flow, which is associated with cardioprotection, in perfused hearts (Feng, et al., 2002; Garlid, et al., 1997; Suzuki, et al., 2003), despite the fact that the coronary flow reserve is low in crystalloid-perfused hearts (Deng, et al., 1995; Masuda, et al., 1994). In a study with barbiturate-anesthetized dogs, administration of diazoxide (2.5mg/kg) prior to 30 min of regional ischemia and 1h reperfusion almost doubled the transmural myocardial perfusion in the ischemic region

(measured with labeled microspheres) (Alcindor, et al., 2004). Thus, improved cardiac perfusion (local or global) may possibly account for at least some of the cardioprotective effects of diazoxide.

### Does diazoxide activate 'cardiac' sarcolemmal $K_{ATP}$ channels?

Before addressing this question, it would be instructive to examine the mechanism by which diazoxide opens  $K_{ATP}$  channels.  $K_{ATP}$  channels are heterooctamers of four Kir6.1 and/or Kir6.2 subunits in complex with SURx subunits (Nichols, 2006). Two genes (*ABCC8* and *ABCC9*) respectively give rise to SUR1 and SUR2 subunits, each of which may be alternatively spliced. The most commonly described splice variants studied are SUR1, SUR2A and SUR2B (Nichols, 2006). When comparing the effects of diazoxide on Kir6.2/SUR1, Kir6.2/SUR2A or Kir6.2/SUR2B channels, it was found that the effects of diazoxide depend on the cytosolic ADP levels (Matsuoka, et al., 2000). Each of the subunit combinations were activated by diazoxide, but the activation of Kir6.2/SUR2A was only observed at elevated ADP levels. Thus, under basal conditions and when intracellular ADP levels are low, diazoxide potentially activates SUR1-containing channels (such as the pancreatic  $\beta$ -cell  $K_{ATP}$  channel) or channels with SUR2B (smooth muscle  $K_{ATP}$  channel), which would explain the drug's history of acting on these channels. The inability of diazoxide to activate Kir6.2/SUR2A (the prototypic cardiac  $K_{ATP}$  channel combination (Babenko, et al., 1998)) under basal conditions has also been observed by others (Garlid, et al., 1993), which led to the erroneous consensus that cardiac  $K_{ATP}$  channels are insensitive to diazoxide. At an elevated cytosolic ADP level of 100  $\mu$ M, the SUR2A/Kir6.2 channel attains the same diazoxide sensitivity as the Kir6.2/SUR1 channel. Moreover, cardiac  $K_{ATP}$  channels are readily activated by diazoxide in the presence of a creatine kinase inhibitor (which elevates cytosolic ADP) (D'Hahan, et al., 1999). Since cytosolic ADP levels are substantially elevated during early ischemia (Ramani, et al., 1996), the possibility must be considered that cardiac  $K_{ATP}$  channels are *de facto* effectors of diazoxide during the ischemic period and that they may participate in the drug's cardioprotective actions. Indeed, diazoxide leads to more pronounced shortening of the action potential during the first 10 min of ischemia in an isolated rat heart (Garlid, et al., 1997; Suzuki, et al., 2003) and  $K_{ATP}$  channels open more readily during metabolic inhibition in myocytes pre-incubated with diazoxide (Rodrigo, et al., 2004). Moreover, the preconditioning-like protection of diazoxide is prevented by HMR-1883 (Birincioglu, et al., 1999; Suzuki, et al., 2003; Tanno, et al., 2001), a blocker with putative selectivity for 'the cardiac sarcolemmal  $K_{ATP}$  channel' (Gogelein, et al., 1998) (but see (H. X. Zhang, et al., 2011)). Also, the ability of diazoxide to improve post-ischemic functional recovery is lost in Kir6.2 deficient mice (Suzuki, et al., 2003; Wojtovich, et al., 2013), which lack sarcolemmal  $K_{ATP}$  channels (Suzuki, et al., 2001). These arguments provide strong evidence that sarcolemmal  $K_{ATP}$  channels are diazoxide effectors. Apart from the fact that diazoxide may affect Kir6.2/SUR2A channels (under ischemic conditions), recent progress has questioned the contention that 'the' cardiac  $K_{ATP}$  channel is necessarily comprised of these two subunits. There is a report of a novel diazoxide-sensitive 22 pS conductance  $K_{ATP}$  channel in rat ventricular myocytes (Wu, et al., 2007).  $K_{ATP}$  channels in rodent atria (Baron, et al., 1999; Flagg, et al., 2008), human ventricle (Fedorov, et al., 2011) and the rodent cardiac conduction system (Bao, et al., 2011) all have a high sensitivity to diazoxide (presumably due to the presence of SUR1 or SUR2B) and the contribution of these channels to cardioprotection and arrhythmias should be considered when assessing effectors of diazoxide.

### Diazoxide activates mitochondrial $K_{ATP}$ channels

A  $K^+$  selective ion channel, blocked by ATP with an  $IC_{50}$  of 800  $\mu$ M, was initially described in the inner membrane of fused giant mitoplasts prepared from rat liver mitochondria using

patch clamp methods (Inoue, et al., 1991). A similar mitochondrial  $K_{ATP}$  ( $mK_{ATP}$ ) channel was recorded when purified membranes of rat liver or bovine heart were reconstituted in lipid bilayer membranes (Bednarczyk, et al., 2005; Nakae, et al., 2003; Paucek, et al., 1992; D. X. Zhang, et al., 2001). Patch clamping of the inner mitochondrial membrane of human T-lymphocytes also demonstrated the existence of a  $mK_{ATP}$  channel that is blocked by millimolar concentrations of ATP (Dahlem, et al., 2004). Some studies, however, failed to find  $mK_{ATP}$  channels in the mitochondrial inner membrane (Antonenko, et al., 1994; Brustovetsky, et al., 2005). Despite initial reports suggesting the presence of  $K_{ATP}$  channel subunits (Kir6.x and SURx) in mitochondria, the current consensus appears to be that these subunits do not comprise the  $mK_{ATP}$  channel (Wojtovich, et al., 2013; H. Zhang, et al., 2009). Recent developments implicate novel, short-form, SUR2 splice variants and Kir1.1 subunits as potential subunit candidates (Foster, et al., 2012; Ye, et al., 2009). The  $mK_{ATP}$  channel is activated by the cromakalim analog EMD60480 in the nanomolar range ( $EC_{50}$  of 6 nM) and diazoxide in the micromolar range (2.3–27  $\mu$ M; Table 2; also see (Nakae, et al., 2003)). Other patch clamp data, however, failed to demonstrate activation of  $mK_{ATP}$  channels by 50  $\mu$ M diazoxide (Dahlem, et al., 2004). Diazoxide (10  $\mu$ M) directly increases  $K^+$  fluxes in isolated mitochondria, as measured with the surrogate monovalent flux carrier, thallium ( $Tl^+$ ) (Wojtovich, et al., 2013). The diazoxide concentrations needed to open  $mK_{ATP}$  channels are within the cardioprotective range (Table 1) and are similar to that needed to open other  $K_{ATP}$  channels (Table 2). The activation of  $K_{ATP}$  channel opening by diazoxide can be antagonized with 5-hydroxydecanoate (5HD) (Nakae, et al., 2003; D. X. Zhang, et al., 2001), which is therefore often employed as a  $mK_{ATP}$  channel-selective blocker (Garlid, et al., 1997). However, care should be employed when using this compound since some studies report no (or an unusually slow and irreversible) block of  $mK_{ATP}$  channel by 5HD (Choma, et al., 2009; Dahlem, et al., 2004). Moreover, 5HD may have other off-target effects (e.g., cardiac mitochondria can directly metabolize 5HD (Hanley, et al., 2002; Suleiman, et al., 2001)). The selectivity of diazoxide to affect only  $mK_{ATP}$  channels is increasingly being questioned (Jaggar, et al., 1993; Li, et al., 2010; Moritani, et al., 1994; Notsu, Ohhashi, et al., 1992; Notsu, Tanaka, et al., 1992; K. Sakamoto, et al., 1998; Szewczyk, et al., 2010). Given the electrogenic nature of the  $mK_{ATP}$  channel, the expectation is that the mitochondrial membrane potential ( $\Delta\psi_m$ ) may be affected by its opening. Mixed results exist in this regard, with some results indicating no effect, with others suggesting depolarization of the  $\Delta\psi_m$  (Table 3). Interestingly, the depolarizing effect of 10  $\mu$ M, but not 100  $\mu$ M diazoxide, is blocked by 5-hydroxydecanoic acid (5HD), which the authors interpreted as non-specific effects at the higher diazoxide concentrations (Murata, et al., 2001). A similar conclusion was reached by others, suggesting that at  $>50 \mu$ M, diazoxide has mitochondrial effects that are independent of  $mK_{ATP}$  channels (Kowaltowski, et al., 2001). Interestingly,  $\Delta\psi_m$  depolarization induced by anoxia/reoxygenation or reactive oxidant species is prevented by diazoxide (Table 3). Since maintenance of  $\Delta\psi_m$  is essential for ATP synthesis, this may be a potential protective or anti-ischemic effect.

## Diazoxide improves mitochondrial function

As early as 1969, diazoxide has been recognized as an inhibitor of the mitochondrial complex II protein, succinate dehydrogenase (SDH) (Schafer, et al., 1969). This inhibition, which is also observed in heart (Hanley & Daut, 2005; Hanley, et al., 2002), occurs at concentrations often used to study cardioprotection ( $K_{1/2}=32 \mu$ M; Table 1) and similar to those affecting other diazoxide effectors (Table 2). As expected, SDH inhibition by diazoxide leads to increased flavoprotein fluorescence (Schafer, et al., 1971) (but see (Lawrence, et al., 2001)) with a  $K_{1/2}$  of 27  $\mu$ M (Y. Liu, et al., 1998). It is possible that the cardioprotective effect of diazoxide is due at least partly to SDH inhibition since other SDH inhibitors (e.g. thenoyltrifluoroacetone) are also cardioprotective (Duda, et al., 2007). The mechanisms by which SDH inhibition is cardioprotective are under active investigation and

may relate to partial uncoupling of oxidative phosphorylation (Kopustinskiene, et al., 2003),<sup>53</sup>. Indeed, short-term administration of 2,4-dinitrophenol (DNP), an uncoupler of oxidative phosphorylation, induces preconditioning-like cardioprotection (Minners, et al., 2000), an effect that is independent of  $K_{ATP}$  channel activation (Rodrigo, et al., 2002). Since the mitochondrial effects of diazoxide are dependent on the metabolic substrate used and do not persist in the absence of  $K^+$  (B. Liu, et al., 2010; Riess, et al., 2008), this also argues against a  $K_{ATP}$  channel-mediated response. Experiments with other approaches further demonstrated that transient (or partial) inhibition of the oxidative phosphorylation may serve as a mechanism to reduce I/R injury (Stottrup, et al., 2010). The activity of SDH, coupled with the coenzyme Q cycle, is a site of generation of reactive oxygen species (Demin, et al., 1998) and the possibility has been raised that diazoxide and other SDH inhibitors mediate (at least some of) their cardioprotective effects via modulation of ROS production (Carroll, et al., 2001; Drose, et al., 2011; Dzeja, et al., 2003; Pain, et al., 2000; Pasdois, et al., 2008; Terzic, et al., 2000). Additionally, diazoxide has been demonstrated to protect mitochondrial energetic function and prevent swelling during metabolic stress (Dos Santos, et al., 2002; Iwai, et al., 2000; Ozcan, et al., 2001), partly by preventing  $Ca^{2+}$ -dependent inhibition of oxidative phosphorylation (Holmuhamedov, et al., 2012). Whatever the underlying molecular mechanism(s), it is clear that improved mitochondrial function must be considered as an important diazoxide effector in cardioprotection.

### Are endothelial $K_{ATP}$ channels involved?

Although diazoxide can cause vasodilation in the absence of an intact endothelium (Antoine, et al., 1992), several reports indicate the endothelium to be another diazoxide target (Feleder & Adler-Graschinsky, 1997). In humans, plethysmography recordings of forearm blood flow demonstrated that endothelial function is impaired by ischemia (induced by blood pressure cuff inflation), which is prevented by IPC or diazoxide pre-administration (800  $\mu\text{g}/\text{min}$  for 20 min) (Broadhead, et al., 2004). Post-ischemic endothelial dysfunction also occurs in animal models, and can be minimized by IPC or diazoxide-preconditioning (Duda, et al., 2006; Pagliaro, et al., 2003). Although excessively high diazoxide concentrations were used in some of these studies, the coronary endothelium is in fact exquisitely sensitive to diazoxide: it causes endothelial hyperpolarization in the sub-micromolar concentration range and  $Ca^{2+}$  oscillations have been reported to occur with concentrations as low as 100 nM (Langheinrich, et al., 1998; White & Hiley, 2000).  $K_{ATP}$  channels in the endothelium are established to contribute significantly to coronary function (Ishizaka & Kuo, 1997; Janigro, et al., 1993; Kuo & Chancellor, 1995; Q. Liu & Flavahan, 1997; Malester, et al., 2007; Schnitzler, et al., 2000; von Beckerath, et al., 1996). The endothelial  $K_{ATP}$  channels are thought to be composed of Kir6.1 and Kir6.2 in combination with the diazoxide-sensitive SUR2B subunit (Jansen-Olesen, et al., 2005; Schnitzler, et al., 2000; Yoshida, et al., 2004) (Table 2). Activation of these channels by diazoxide (Schnitzler, et al., 2000) may be partly responsible for the observed electrophysiological effects. It is unclear at present how diazoxide leads to protection against endothelial dysfunction. Mitochondrial energetics and  $mK_{ATP}$  channel activation may contribute, but the changes in membrane potential and cytosolic  $Ca^{2+}$  suggest that other ion channels (particularly endothelial  $K_{ATP}$  channels) may also be involved.  $K_{ATP}$  channel-mediated endothelial hyperpolarization may be propagated to the coronary smooth muscle through connexin-mediated myo-endothelial electrical communication (Murai, et al., 1999). It is also possible that endothelial  $K_{ATP}$  channel opening (and associated changes in cytosolic  $Ca^{2+}$ ) may benefit the secretion of vasoactive substances such as nitric oxide (Luckhoff & Busse, 1990; H. Wang, et al., 2007), which has an established cardioprotective role (Downey, et al., 2007). It should be a priority of future experiments to investigate these possibilities.

## Diazoxide regulates neurotransmitter release

A novel role for  $K_{ATP}$  channels has been described in the sympathetic nervous system, where it was found that norepinephrine (NE) release is inhibited by active  $K_{ATP}$  channels (Oe, et al., 1999). The data supporting this finding are that  $K_{ATP}$  channel openers (including 100  $\mu$ M diazoxide) inhibit NE release as well as the increase in atrial rate induced by electrical stimulation of the sympathetic ganglion (Mohan & Paterson, 2000; Oe, et al., 1999). In contrast,  $K_{ATP}$  channel blockers had the opposite effects.  $K_{ATP}$  channels also are involved in neurotransmitter release from parasympathetic nerves. Acetylcholine (ACh) is synthesized and released in the central nervous system as well as from autonomic ganglia in the peripheral nervous system and in postganglionic parasympathetic neurons. In the heart, vagal nerve stimulation causes ACh release, which slows the heart rate by G-protein-mediated activation of Kir3.x channels (Coetzee, et al., 1999). It was found that ACh-release evoked by electrical stimulation of isolated guinea pig atria was stimulated by  $K_{ATP}$  channel blockers, suggesting a role for negative feedback of  $K_{ATP}$  channels in exocytotic processes in these neurons (Kilbinger, et al., 2002). Importantly, 30  $\mu$ M diazoxide significantly reduced the stimulation-evoked release of [ $^3$ H]acetylcholine. Interestingly, ACh release of neurons in the ileum is affected similarly by  $K_{ATP}$  channel activity (Zini, et al., 1991), whereas mesenteric neurons are not affected by  $K_{ATP}$  channel modulation (Kilbinger, et al., 2002; Schworer & Kilbinger, 1989). These observations suggest diversity of  $K_{ATP}$  channels in various neuronal compartments, which remains to be studied. The  $K_{ATP}$  channels in dorsal vagal neurons have been suggested to be composed of Kir6.2/SUR1 channels (Karschin, et al., 1998) (as in the pancreas), which raises issues of potential cardiovascular side-effects of sulphonylurea treatment in the setting of diabetes. The study of the molecular composition of the  $K_{ATP}$  channels in sympathetic and parasympathetic neurons and the molecular mechanisms responsible for coupling  $K_{ATP}$  channel activity to exocytotic release await more refined tools (such as mice lacking  $K_{ATP}$  channels in specific tissue compartments). Moreover, the pathophysiological consequences of modulating neurotransmitter release by diazoxide in the context of ischemia and cardioprotection remain a fertile area for future investigation. Interestingly, depletion of presynaptic nerve terminals of their NE stores with reserpine (a monoamine transporter blocker) results in the failure of ischemic preconditioning protocols to be cardioprotective (Toombs, et al., 1993), suggesting that the NE release is critical to preconditioning-mediated protection. The ability of diazoxide to stimulate NE release from sympathetic nerve endings must therefore be considered when evaluating the cardioprotective actions of the compound.

## Other diazoxide effectors

Other than those discussed above, diazoxide affects the functions of several other proteins and biological processes. For example, in the cardioprotective range, 100  $\mu$ M diazoxide causes a significant increase in ATPase activity in purified cardiac membranes isolated from guinea pig hearts (Bienengraeber, et al., 2000). Stimulation of mitochondrial ATPase activity has also been reported (Dzeja, et al., 2003; Portenhauser, et al., 1971), which is due to stimulation of the  $F_0F_1$  ATP synthase (Belisle & Kowaltowski, 2002). The mechanism appears to be that diazoxide stabilizes Mg-ADP bound in the catalytic site (possibly within the nucleotide binding fold) of the  $\epsilon$  subunit of the mitochondrial ATP synthase (Contessi, et al., 2004). A similar mechanism has been reported for activation of  $K_{ATP}$  channels by diazoxide (Bienengraeber, et al., 2000). Thus, the possibility should be considered that stabilization of Mg-ADP complexes at nucleotide binding folds might be a general action of diazoxide.

Diazoxide also affects other ion channels. For example, inhibition of voltage gated inward and outward currents has been reported in CA1 hippocampal neurons (Erdemli & Krnjevic,

1995). Direct effects of diazoxide on cardiovascular ion channels have not yet been reported. Interestingly, however, pharmacological preconditioning with 100  $\mu\text{M}$  diazoxide strongly reduces the cardiac L-type  $\text{Ca}^{2+}$  channel density and the amplitude of cytosolic  $\text{Ca}^{2+}$  transients (Gonzalez, et al., 2010). It remains to be determined to what extent cardiovascular ion channels (other than  $\text{K}_{\text{ATP}}$  channels) are affected by diazoxide-induced preconditioning. There are also reports that diazoxide may act to open the mitochondrial permeability transition pore (Kato, et al., 2002). Non-channel mediated actions of diazoxide have also been reported. For example, 100  $\mu\text{M}$  diazoxide was reported to induce translocation of PKC- $\beta$  from the cytosol to mitochondria in H9c2 cells. Moreover, diazoxide-induced flavoprotein oxidation was inhibited by PKC- $\beta$  inhibition and transfection with wild type PKC- $\beta$  mimicked the flavoprotein-oxidizing effect of diazoxide). These data led the authors to conclude that the primary effect of diazoxide is PKC- $\beta$  activation (Kim, et al., 2006), which has known cardioprotective properties (Steinberg, 2012). Diazoxide also attenuates swelling of isolated rabbit ventricular myocytes during metabolic inhibition (Al-Dadah, et al., 2007).

## Conclusion

A drug does not have to be selective in order to be effective. It has been argued that an ideal drug may be one whose efficacy is not solely based on a single target, but rather on rebalancing several biological effectors/process that contribute to the etiology, pathogenesis, and progression of diseases (i.e. a promiscuous drug) (Mencher & Wang, 2005). Diazoxide appears to be such a drug: while it is clear that diazoxide has potent cardioprotective properties, a review of the literature shows that diazoxide has multiple effectors that may synergistically contribute to its cardioprotective properties (Figure 2). Diazoxide is, however, neither specific nor selective. The specificity of diazoxide (defined here as the capacity of a drug to have a unique action) is questionable since it affects blood pressure, blood glucose levels and contributes to cardioprotection. Diazoxide also lacks selectivity (defined here as the ability of a drug to affect a particular molecular target in preference to others) since it affects several types of  $\text{K}_{\text{ATP}}$  channels and also have other off-target effects. Moreover, the diazoxide sensitivities of these various effectors are similar (and within the range of concentrations used for most studies when examining cardioprotection). Care should be taken when referring to diazoxide as having specificity or selectivity to a particular biological process or effector (e.g. an ion channel) and the reader is urged to consider the multiplicity of diazoxide's effectors when evaluating the cardioprotection literature.

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## List of abbreviations

<b>m</b>	mitochondrial membrane potential
<b>5HD</b>	5-hydroxydecanoate
<b>DNP</b>	2,4-dinitrophenol

<b>I/R injury</b>	Ischemia/reperfusion injury
<b>IPC</b>	Ischemic preconditioning
<b>K<sub>ATP</sub> channel</b>	ATP-sensitive K <sup>+</sup> channel
<b>ROS</b>	Reactive oxygen species
<b>SDH</b>	succinate dehydrogenase

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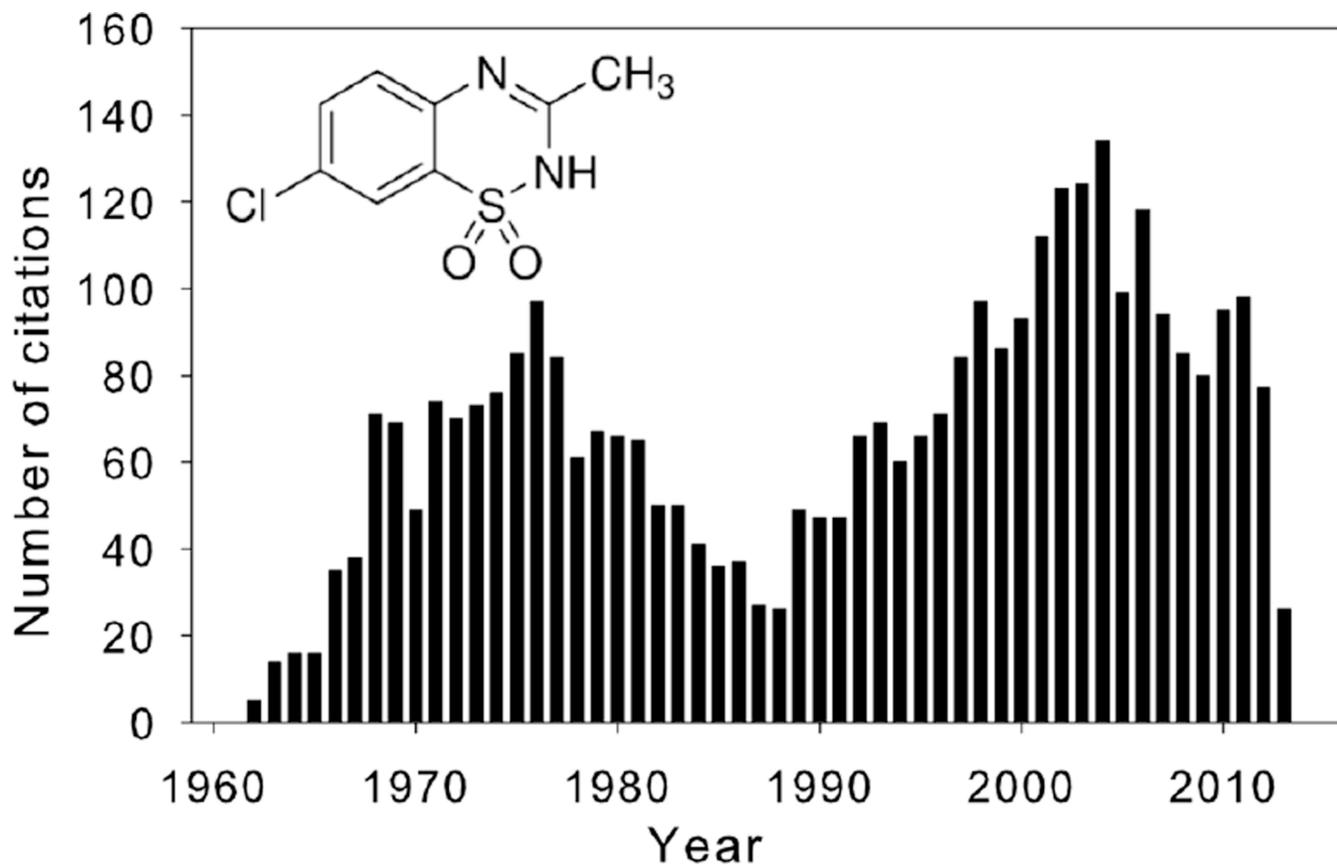
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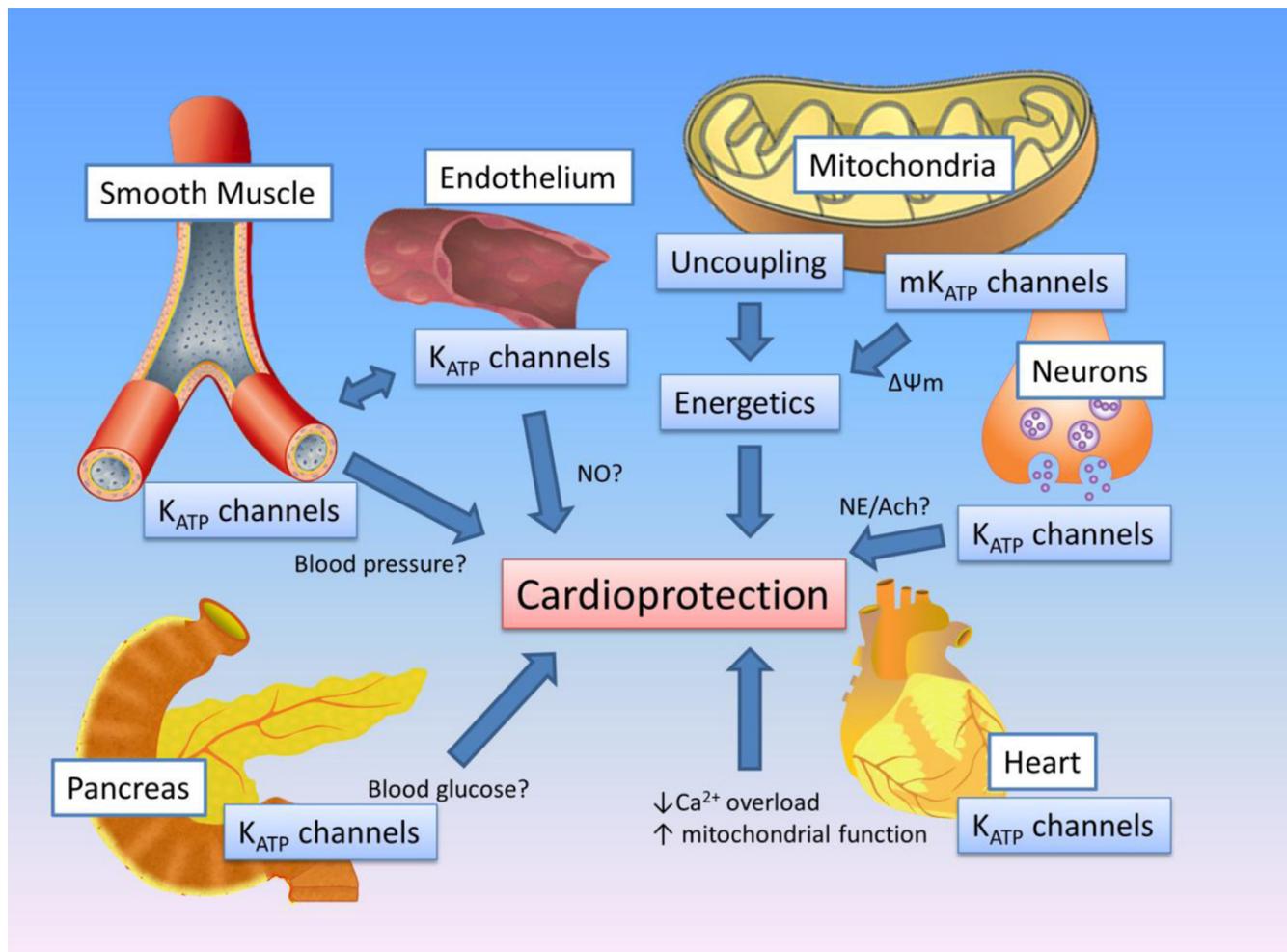
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**Figure 1.** The number of PubMed citations returned when searching with the keyword “diazoxide”. The data are binned over the time period 1960–2013. The inset shows the structural formula for diazoxide.



**Figure 2.**

A cartoon summary of the main potential effectors of diazoxide, depicting some of the potential mechanisms by which they may contribute to cardioprotection. NO is nitric oxide, NE is norepinephrine and Ach is acetylcholine. For details and concentrations at which diazoxide affects these targets, please refer to the text and the Tables.

Table 1

## Diazoxide is cardioprotective

Preparation	Procedure	Result	Concentration
Isolated rat hearts	25 minutes of global ischemia and 30 minutes of reperfusion	Diazoxide and cromakalim increased the time to onset of contracture and improved post-ischemic functional recovery	11–30 $\mu$ M (Garlid, et al., 1997)
<i>In vivo</i> rabbits	30 min of regional ischemia and 3 h of reperfusion	Diazoxide administered before ischemia reduces infarct size	10 mg/kg (Baines, et al., 1999)
Langendorff-perfused rat hearts	Diazoxide pretreatment prior to 40 minutes ischemia and 30 minutes of reperfusion	Diazoxide improved left ventricular end-diastolic pressure, LDH release and coronary flow after I/R	1–100 $\mu$ M (80 $\mu$ M is optimal) (Y. Wang, et al., 1999)
Langendorff-perfused rabbit hearts	Diazoxide pretreatment prior to 30 minutes ischemia and 60 minutes of reperfusion	Reduction in infarct size and improved mitochondrial function	100 $\mu$ M (Miura, et al., 2000)
<i>In vivo</i> rabbits	30 min of regional ischemia and 3 h of reperfusion	Diazoxide administered before ischemia reduces infarct size	1 mg/kg (Ockaili, et al., 1999)
<i>In vivo</i> rats	30 min of regional ischemia and 2 h of reperfusion	Diazoxide administered before ischemia reduces infarct size	10 mg/kg iv (Fryer, et al., 2000)
Human right atrial specimens	90 min 'ischemia' and 2h 'reperfusion'	Less CK leakage in diazoxide group	100 $\mu$ M (Ghosh, et al., 2000)
Isolated rabbit hearts	30 min of regional ischemia and 2 h of reperfusion	Diazoxide administered before ischemia reduces infarct size	100 $\mu$ M (S. Wang, et al., 2001)
Isolated mouse hearts	20 min of global ischemia and 1 h of reperfusion	Improved post-ischemic functional recovery	100 $\mu$ M (Suzuki, et al., 2003)
Isolated mouse hearts	30 min of global ischemia and 1 h of reperfusion	Diazoxide administered before ischemia improved functional recovery and reduced infarct size. The protection was blunted in Kir6.2(–/–) mice	30 $\mu$ M (Wojtovich, et al., 2013)
Isolated mouse hearts	40 min of global ischemia and 30 minutes of reperfusion	Diazoxide IV administration 24 h prior to ischemia improves post-ischemic functional recovery	7 mg/kg (Y. Wang, et al., 2001)
Open-chest pig	30 min of regional ischemia and 3 h of reperfusion	Diazoxide administered before ischemia reduces infarct size	3.5 mg/kg (Schwartz, et al., 2002)
Open-chest beagle dogs	90 min of regional ischemia and 6 h of reperfusion	Diazoxide partially decreased infarct size	80 $\mu$ M (Sanada, et al., 2001)

**Table 2**

## Effectors of diazoxide

Target	Effect	Concentration ( $K_{1/2}$ )
Mitochondrial $K_{ATP}$ channel	Opens	2–27 $\mu$ M (Garlid, et al., 1993; Y. Liu, et al., 2001; Wojtovich, et al., 2013)
Pancreatic $\beta$ -cell $K_{ATP}$ channel	Opens	7–20 $\mu$ M (Trube, et al., 1986; Zunkler, et al., 1988)
Kir6.1/SUR1 or Kir6.2/SUR1 channels	Opens	10 $\mu$ M (Y. Liu, et al., 2001)
Smooth muscle	Relaxation	7–20 $\mu$ M (Mahmoudian & Mirkhani, 1998; Newgreen, et al., 1990)
Rabbit mesenteric $K_{ATP}$ channel	Opens	37 $\mu$ M (Quayle, et al., 1995)
Kir6.1/SUR2B channels	Opens	60 $\mu$ M (Inagaki, et al., 1995)
Ventricular $K_{ATP}$ channels	Cytosolic ADP is needed (D'Hahan, et al., 1999)	
Kir6.2/SUR2A channels	Cytosolic ADP is needed (D'Hahan, et al., 1999; Matsuoka, et al., 2000)	
Succinate dehydrogenase	Inhibition	32–49 $\mu$ M (Dzeja, et al., 2003; Schafer, et al., 1969)

**Table 3**

Effects of diazoxide on mitochondrial membrane potential ( m)

Preparation	Concentration	Method	Result
Isolated rat cardiac myocytes	200 μM	TMRE fluorescence	No effect on m (Lawrence, et al., 2001)
Isolated guinea-pig ventricular myocytes	100 μM	TMRE fluorescence	No effect on m (Hanley, et al., 2002)
Cultured human atrial-derived cardiocytes	30 μM	JC-1 fluorescence	No effect on m (Carroll, et al., 2001)
Cultured hippocampal neurons	30 μM	TMRE fluorescence	Depolarize m (D. Liu, et al., 2002)
Isolated mitochondria from piglet brain	50–500 μM	TMRE fluorescence	Depolarize m (Busija, et al., 2005)
Isolated rat cardiac mitochondria	500 μM	TMRE fluorescence	Depolarize m (Katakam, et al., 2007)
Permeabilized rabbit ventricular myocytes	10–100 μM	TMRE fluorescence	Depolarize m (Murata, et al., 2001)
Rat heart mitochondria	>50 μM	TPP <sup>+</sup> -selective electrode	Depolarize m (Kowaltowski, et al., 2001)
Isolated rat cardiac myocytes	100 μM	TMRE fluorescence	<sup>a</sup> Prevents ROS-mediated m depolarization (Jin, et al., 2012)
Neonatal rat cardiac myocytes	20–200 μM	TMRE fluorescence	Prevents H <sub>2</sub> O <sub>2</sub> -mediated m depolarization (Akao, et al., 2001)
Cultured neonatal rat cardiac myocytes	100 μM	JC-1 fluorescence	Protects against m depolarization induced by anoxia/reoxygenation (Xu, et al., 2001)
Cultured H9c2 cells	50 μM	TMRE fluorescence	Prevents m depolarization induced by hypoxia/reoxygenation (Mykytenko, et al., 2008)

<sup>a</sup>The intervention used was intermittent laser illumination of a 15 min period.