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K^+-Channel blockers and coronary vasoconstriction in guinea-pig perfused hearts in-vitro

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Abstract—Glibenclamide, glipizide and phentolamine, three drugs which have been reported to block ATP-dependent potassium channels, increased the coronary perfusion pressure in guinea-pig isolated hearts perfused at constant flow. Blockers of other types of potassium channels, 4-aminopyridine and UK-66,914, did not significantly increase perfusion pressure. Exposing hearts to a single concentration of 3 μM glibenclamide caused a greater degree of vasoconstriction than when this was preceded by lower concentrations. The 3 μM glibenclamide-induced vasoconstriction was reduced by prazosin (1 μM), mepyramine (0·1 μM) and ranitidine (10 μM) but not by a combination of mepyramine and ranitidine or by ritanserin (0·01 μM). These results suggest that a component of the vasoconstriction induced by glibenclamide may result indirectly from the release of vasoactive mediators.

Glibenclamide is known to block potassium channels in a variety of tissue types including vascular tissues (Winquist et al 1989; Ksoll et al 1991). In isolated vascular preparations, this blockade is manifested as a prevention or reversal of the vasorelaxant effects of potassium-channel opening drugs (Cavero et al 1988; Winquist et al 1989; Satoh et al 1991). Glibenclamide itself has been observed in some studies to exert little vasoconstrictor effect on vascular tone when applied at similar concentrations in the absence of potassium-channel openers (Daut et al 1990; Satoh et al 1991; Zhang et al 1991), but some reports (Wolleben et al 1989; Wilde et al 1990; Mitani et al 1991) have described a marked but unexplained coronary vasoconstriction following the application of glibenclamide to rat and guinea-pig isolated hearts in-vitro. We have investigated this phenomenon in guinea-pig isolated hearts perfused at constant flow. The actions of glibenclamide have been compared with those of glipizide and phentolamine, as all of these agents have been reported to block ATP-sensitive K^+ channels (K_{ATP}) (Fosset et al 1988; Plant & Henquin 1990). For comparison, we have also studied other K^+-channel blockers, UK-66,914, which blocks delayed rectifier type potassium channels (Gwilt et al 1991) and 4-aminopyridine, a relatively non-selective potassium-channel blocker (Glover 1982).

Materials and methods

Experimental preparation. Hearts were prepared as described previously (Gwilt et al 1992). Briefly, male guinea-pigs were injected with heparin (1000 units, i.p.), and killed 30 min later by concussion followed by exsanguination. Hearts were excised and placed in well-oxygenated (95% O_2–5% CO_2) ice-cold physiological buffer solution of the following composition (mM): NaCl 118, KCl 4·7, $MgSO_4$ 1·17, $NaHCO_3$ 25, KH_2PO_4 1·2, D-glucose 2·5, $CaCl_2$ 2·5. Hearts were cannulated via the aorta (Langendorff technique), and perfusion (32°C) was started with an initial flow of 6 mL min^{-1} to clear hearts of blood (about 5 min). Gradually the flow rate was increased until the perfusion pressure reached 50 mmHg and was left for the remainder of the experiment. Platinum electrodes were implanted into the preparation for stimulation (Grass S88, Quincey, MA, USA) at 5 ms duration, 3 × diastolic threshold and 4 Hz.

Hearts were allowed to equilibrate for 10–15 min and the actual perfusion pressure at the start of the experimental protocol was noted (see Results). K^+-Channel blockers were then added to the superfusate and perfusion pressure monitored for 30 min. In other experiments, receptor antagonists were added to the superfusate and 30 min later glibenclamide (3 μM) was also added and hearts were perfused with the drug plus glibenclamide mixture for a further 30 min. The perfusion pressure was measured immediately before the addition of glibenclamide and the highest pressure achieved during the perfusion with glibenclamide was noted. Alternatively, in other experiments, increasing concentrations of glibenclamide were added cumulatively to the perfusate at 30 min intervals without the addition of other drugs.

Drugs. Glibenclamide and 4-aminopyridine were from Sigma Chemical Co., phentolamine (Rogitine) was purchased from Ciba and glipizide was obtained from Carlo Erba; other drugs were synthesized in-house. All drugs were dissolved in 80% dimethylsulphoxide 20% buffer to form a 10^{-2} M stock solution which was diluted further in the same solvent as appropriate.

Presentation of data. Data are presented as means ± s.e. The significance of vasoconstrictor effects of glibenclamide were determined using *t*-tests to compare changes in the presence of drugs with corresponding changes in vehicle-treated tissues (except where indicated) after an exploratory analysis of variance to highlight significant differences within each treatment group. The level of significance was taken as $P < 0.05$.

Results

The dimethylsulphoxide vehicle used in these experiments did not induce significant changes in perfusion pressure (Table 1). In contrast, marked vasoconstriction was observed following the application of a single concentration of 3 μM glibenclamide to each heart. The peak of the increase in pressure occurred between 7 and 14 min after the addition of glibenclamide and slowly declined thereafter. After 30 min exposure to glibenclamide, the remaining increase in perfusion pressure was no longer significantly different from control. Similar increases in perfusion pressure were observed following the application of other putative K_{ATP}-channel blockers. Glipizide, a sulphonylurea structurally related to glibenclamide (Fosset et al 1988), increased the perfusion pressure at a concentration of 30 μM, a lower concentration of 3 μM being without significant effect. Phentolamine also increased the perfusion pressure at a concentration of 3 μM. The vasoconstriction due to glipizide was better maintained than that due to the other agents, as a significant increase in pressure was still present at the end of the 30-min experimental period. UK-66,914 and 4-aminopyridine did not significantly increase coronary perfusion pressure.

In further experiments the mechanism of this phenomenon was explored using glibenclamide, as this is the most frequently studied K_{ATP}-channel blocker. Fig. 1 shows the average in-

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Table 1. Effects of potassium-channel blockers on coronary perfusion pressure.

Coronary perfusion pressure (mmHg)	Dimethylsulphoxide (7)	Glibenclamide 3 μM (10)	Glipizide 3 μM (4)	Glipizide 30 μM (5)	Phentolamine 3 μM (4)	UK-66,914 10 μM) (5)	4-Aminopyridine 30 μM (6)
Pre-drug	51±2	50±0·4	44±2	51±2	49±1	52±5	47±3
Peak	55±2	80±4***	55±4	86±7**	75±10*	63±9	50±5
30 min	51±3	60±3	55±4	81±11*	70±12	63±9	48±4

Means±s.e. of coronary perfusion pressure before addition of drugs or vehicle, the highest values during perfusion with drugs and values after 30 min of perfusion. *$P<0·05$, **$P<0·01$, ***$P<0·001$, compared with pre-drug values.
Numbers in parentheses denote number of experiments.

creases in perfusion pressure induced by glibenclamide applied cumulatively in the perfusate. The application of 0·1 μM glibenclamide induced large changes in perfusion pressure in two of the six hearts. The extent of the increases were comparable with those observed in the experiments described above in which a single concentration of 3 μM was applied. The responses to further concentrations of glibenclamide varied in magnitude, but overall perfusion pressure remained elevated by 15–50 mmHg throughout the experiment in these preparations. In the other four hearts, the perfusion pressure was essentially unaffected, remaining within 4 mmHg of that before glibenclamide administration despite the application of concentrations of glibenclamide as high as 10 μM. Therefore, in contrast to the earlier experiments where the application of a single concentration of 3 μM induced a large change in perfusion pressure in all preparations studied, a more gradual increase in the concentration of glibenclamide up to and above this concentration usually resulted in little or no increase in pressure.

Potential mechanisms of the vasoconstriction were explored using a range of receptor antagonists (Table 2). In contrast to the marked and significant effect of glibenclamide alone on perfusion pressure, the increase in this parameter following glibenclamide application in the presence of prazosin (1 μM) was not significantly different from changes occurring in the presence of vehicle, despite the vasoconstriction previously observed with the combination of non-selective α-adrenoceptor blockade and K_{ATP} block with phentolamine described above. The glibenclamide response was also reduced by ranitidine (10 μM) and mepyramine (0·1 μM) as the effects of glibenclamide measured in the presence of these agents were also not significantly different from control. Surprisingly, the vasoconstrictor effect of glibenclamide was preserved in the presence of a mixture of ranitidine and mepyramine. While a significant increase in perfusion pressure was induced by the combination of mepyramine and ranitidine before glibenclamide application in this experiment, the increase in perfusion pressure following the addition of glibenclamide was significantly elevated to a level comparable with the effect of glibenclamide alone. Finally, the perfusion pressure was also significantly elevated by glibenclamide in the presence of ritanserin to a similar degree to that seen using glibenclamide alone.

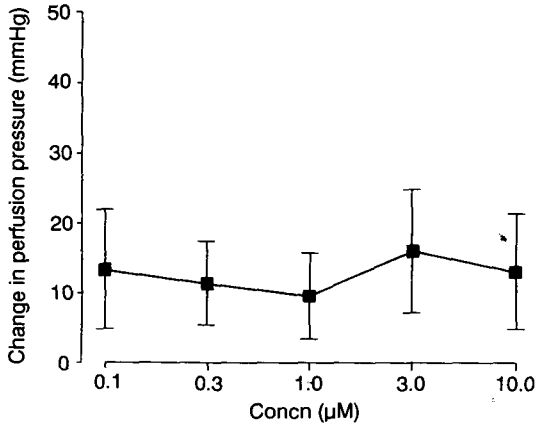

FIG. 1. Changes in coronary perfusion pressure (means±s.e.) during perfusion of six hearts with increasing concentrations of glibenclamide added cumulatively to the superfusate. No increase was significantly different from pre-drug controls.

Discussion

The effects of glibenclamide on the mechanical activity of vascular preparations in-vitro or in-vivo varies considerably between studies. Previous reports have described increases in coronary resistance of rat perfused hearts of approximately 25% following perfusion with glibenclamide at 1 μM (Wolleben et al 1989) and reductions of coronary flow in rat, rabbit and ferret perfused hearts of 38–40% following perfusion with glibenclamide at a concentration of 3 μM (Wilde et al 1990). Other studies have reported no effect of glibenclamide on the coronary vasculature of rat and guinea-pig perfused hearts in-vitro (Daut et al 1990; Grover et al 1990; Kantor et al 1990). While the reason for these differences is unclear, the results of the present study indicate that in our hands the exposure of hearts to 3 μM glibenclamide (without prior exposure of hearts to lower concentrations) reproducibly induced a marked coronary vasoconstriction. The similar effects observed with glipizide and phentolamine, and the lack of effect of the other K^+-channel blockers, suggested that the blockade of a K^+ channel similar to the K_{ATP} channel may be involved in this response, although the site of this channel is not clear (see below).

The experiments involving receptor antagonists suggested that release of vasoactive amines may also have been involved. In guinea-pig hearts H_1 receptors may mediate vasoconstriction of coronary arteries via H_1 receptors mainly on the vascular smooth muscle, while endothelial H_2 receptors mediate vasodilatation (Broadley 1975; Kang et al 1987; Wiest et al 1989), although in some circumstances H_1 receptors may also mediate vasodilation (Broadley 1975). In the present study, the suppression of the glibenclamide-induced vasoconstriction by histamine-receptor antagonists indicated that histaminergic mechanisms may have been involved.

Antagonists of both types of histamine receptor, however, reduced the glibenclamide-induced vasoconstriction, whereas the combination of these agents left the glibenclamide-induced vasoconstriction essentially unaffected. These results suggest

Table 2. Effects of drugs on glibenclamide-induced increases in perfusion pressure in perfused hearts.

		Glibenclamide (3 μM)+				
	Dimethylsulphoxide (7)	Prazosin (1 μM) (7)	Ranitidine (10 μM) (8)	Mepyramine (0·1 μM) (7)	Ranitidine+Mepyramine (10 μM) (5)	Ritanserin (0·01 μM) (5)
Pre-drug	51±2	51±1	51±1	50±2	52±1	49±2
Pre-glibenclamide	51±3	51±1	51±1	53±4	65±2**	59±1
Peak	56±4	66±8	66±6	63±9	87±5**	79±6*

Figures (means±s.e.) represent coronary perfusion pressure (mmHg) before addition of drugs or vehicle, 30 min after addition of antagonists and immediately before glibenclamide administration and the highest pressure attained after subsequent addition of glibenclamide. *$P<0.05$, **$P<0.01$ compared with pre-drug values.
Numbers in parenthesis denote number of experiments.

that the situation was considerably more complex than merely release of histamine by glibenclamide; in such a scenario, based on the effects of exogenous histamine, it would have been expected that the effects of mepyramine and ranitidine would have been opposite in direction, while a combination of these agents would have been expected to suppress the glibenclamide response.

In addition, the glibenclamide-induced vasoconstriction was also reduced by prazosin, an α_1-adrenergic-receptor antagonist. This suggests that release of noradrenaline within the hearts may have also mediated a component of the vasoconstriction. Finally, vasoconstriction mediated by 5-HT did not appear to play an important role in these experiments, as the 5-HT_2 antagonist ritanserin (Janssen 1985) was also without effect.

The actions of histamine and noradrenaline on the coronary vasculature are known to be complex. Histamine has been shown to modulate the release of noradrenaline and prostaglandins from vascular preparations in-vitro (Juan & Sametz 1980; Gross et al 1984) while, conversely, noradrenaline increases histamine release from guinea-pig perfused hearts (Gross et al 1984). A number of other vasoconstrictor and vasodilator substances, such as neuropeptide Y, calcitonin gene-related peptide, endothelin, prostaglandins, endothelium-derived relaxing factor and other mediators, are contained within cardiac nerves and the vascular endothelium (Juan & Sametz 1980; Burnstock 1990; Heller & Regal 1990) and may be released by the application of drugs, neurotransmitter substances and other vasoactive substances (Haass et al 1991).

These results argue against a direct effect of glibenclamide via the blockade of potassium channels in the vascular smooth muscle. Such a mechanism would require the pharmacological blockade of this effect by these different agents, which is unlikely. In addition, it would be difficult to explain the lack of effect of glibenclamide on perfusion pressure in most preparations when the concentration of glibenclamide was raised gradually if a direct effect on vascular smooth muscle was involved. The common vasoconstrictive effect of three agents described as K_{ATP} blockers, however, suggests that the effect may be linked in some way to the blockade of K_{ATP} or similar channels. Potassium-channel blockade is known to induce exocytosis and secretion from a number of secretory cell types so that the stimulation of mediator release from the blockade of potassium channels in nerve or other secretory cells remains a possibility.

In conclusion, these results suggest that the vasoconstrictor effects of glibenclamide are probably, at least in part, mediated indirectly via the release of endogenous vasoactive substances in or near the vessel wall. Further studies willl be required to elucidate the precise site of action of K_{ATP} blockers in producing these effects. Blockers of K_{ATP} channels have attracted much interest as putative antifibrillatory agents (Wolleben et al 1989; Kantor et al 1990), where vasoconstrictor responses are obviously inappropriate to the therapy of myocardial ischaemia. If the glibenclamide-induced vasoconstrictive effect is indeed linked to K_{ATP} blockade then this phenomenon may have important implications for the development and use of such agents.

References

Broadley, K. J. (1975) The role of H_1 and H_2 receptors in the coronary vascular response to histamine of isolated perfused hearts of guinea-pigs and rabbits. Br. J. Pharmacol. 54: 511–521

Burnstock, G. (1990) Local mechanisms of blood flow control by perivascular nerves and endothelium. J. Hyperten. 8 (Suppl. 7): S95–S106

Cavero, I., Mondot, M., Mestre, M., Escande, D. (1988) Haemodynamic and pharmacological mechanisms of the hypotensive effects of cromakalim in rats: blockade by glibenclamide. Br. J. Pharmacol. 95: 643

Daut, J., Maier-Rudolph, W., von-Beckerath, N., Mehrke, G., Gunther, K., Goedel-Meinen, L. (1990) Hypoxic dilatation of coronary arteries is mediated by ATP sensitive potassium channels. Science 247: 1341–1344

Fosset, M., De Weille, J. R., Green, R. D., Schmid-Antomarchi, H., Lazdunski, M. (1988) Antidiabetic sulphonylureas control action potential properties in heart cells via high affinity receptors that are linked to ATP-dependent K^+ channels. J. Biol. Chem. 263: 7933–7936

Glover, W. E. (1982) The aminopyridines. Gen. Pharmacol. 13: 259–285

Gross, S. S., Guo, Z. G., Levi, R., Bailey, W. H., Chenouda, A. A. (1984) Release of histamine by sympathetic nerve stimulation in the guinea pig heart and modulation of adrenergic responses. A physiological role for cardiac histamine? Circ. Res. 54: 516–526

Grover, G. J., Sleph, P. G., Dzwonczyk, S. (1990) Pharmacologic profile of cromakalim in the treatment of myocardial ischemia in isolated rat hearts and anesthetized dogs. J. Cardiovasc. Pharmacol. 16: 853–864

Gwilt, M., Dalrymple, H. W., Burges, R. A., Blackburn, K. J., Dickinson, R. P., Cross, P. E., Higgins, A. J. (1991) Electrophysiologic properties of UK-66,914, a novel class III antiarrhythmic agent. J. Cardiovasc. Pharmacol. 17: 376–385

Gwilt, M., Henderson, C. G., Orme, J. O., Rourke, J. D. (1992) Effects of drugs on ventricular fibrillation and ischaemic K^+ loss in a model of ischaemia in perfused guinea-pig hearts in vitro. Eur. J. Pharmacol. 220: 231–236

Haass, M., Richardt, G., Brenn, T., Schömig, E., Schömig, A. (1991) Nicotine induced release of noradrenaline and neuropeptide Y in guinea-pig heart: role of calcium channels and protein kinase C. Naunyn Schmiedebergs Arch. Pharmacol. 344: 527–531

Heller, L. J., Regal, J. E. (1990) Relationship between alterations in atrial and ventricular histamine content and cardiac function during cardiac anaphylaxis of isolated guinea pig hearts. Int. Arch. Allergy Appl. Immunol. 91: 285–290

Janssen, P. A. (1985) Pharmacology of potent and selective S_2-serotonergic antagonists. J. Cardiovasc. Pharmacol. 7: S2–11

Juan, H., Sametz, W. (1980) Histamine-induced release of arachidonic acid and of prostaglandins in the peripheral vascular bed: mode of action. Naunyn Schmiedebergs Arch. Pharmacol. 314: 183–190

Kang, Y. H., Wei, H. M., Fisher, H., Merill, G. F. (1987) Histamine induced changes in coronary circulation and myocardial oxygen consumption: influences of histamine receptor antagonists. FASEB J. 1: 483–489

Kantor, P. F., Coetzee, W. A., Carmeliet, E. E., Dennis, S. E., Opie, L. H. (1990) Reduction of ischemic K^+ loss and arrhythmias in rat hearts. Effect of glibenclamide, a sulfonylurea. Circ. Res. 66: 478–485

Ksoll, E., Parsons, A. A., Mackert, J. R., Schilling, L., Wahl, M. (1991) Analysis of cromakalim, pinacidil and nicorandil induced relaxation of the 5-hydroxytryptamine precontracted rat isolated basilar artery. Naunyn Schmiedebergs Arch. Pharmacol. 343: 377–383

Mitani, A., Knoshita, K., Fukamachi, K., Sakamoto, M., Kurisu, K., Tsuruhara, Y., Fukumura, F., Nakashima, A., Tokunaga, K. (1991) Effects of glibenclamide and nicorandil on cardiac function during ischemia and reperfusion in isolated perfused rat hearts. Am. J. Physiol. 261: H1864–H1871

Plant, T. D., Henquin, J. C. (1990) Phentolamine and yohimbine inhibit ATP sensitive channels in mouse pancreatic beta cells. Br. J. Pharmacol. 101: 115–120

Satoh, K., Yamada, H., Taira, N. (1991) Differential antagonism by glibenclamide of the relaxant effects of cromakalim, pinacidil and nicorandil on canine large coronary arteries. Naunyn Schmiedebergs Arch. Pharmacol. 343: 76–82

Wiest, E., Trach, V., Dammgen, J. (1989) Removal of endothelial function in coronary resistance vessels by saponin. Basic Res. Cardiol. 84: 469–478

Wilde, A. A. M., Escande, D., Schumacher, C. A., Thuringer, D., Mestre, M., Fiolet, J. W. T., Janse, M. J. (1990) Potassium accumulation in the globally ischemic mammalian heart. Circ. Res. 67: 835–843

Winquist, R. J., Heaney, L. A., Wallace, A. A., Baskin, E. P., Stein, R. B., Gracia, M. L., Kaczorowski, G. J. (1989) Glyburide blocks the relaxation response to BRL 34915 (cromakalim), minoxidil sulphate and diazoxide in vascular smooth muscle. J. Pharmacol. Exp. Ther. 248: 149–156

Wolleben, C. D., Sanguinetti, M. C., Siegl, K. S. (1989) Influence of ATP-sensitive potassium channel modulators on ischaemia induced fibrillation in isolated rat hearts. J. Mol. Cell. Cardiol. 21: 783 788

Zhang, H., Stockbridge, N., Weir, B., Krueger, C., Cook, D. (1991) Glibenclamide reduces vascular smooth muscle constriction produced by prostaglandin $F_{2\alpha}$. Eur. J. Pharmacol. 195: 27–35

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Sequential changes of [^{3}H]cyclic AMP binding in the gerbil brain following transient cerebral ischaemia

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Abstract—Sequential alterations in the binding of [^{3}H]cyclic AMP (cAMP) as an indicator of cAMP-dependent protein kinase (cAMP-DPK) binding activity following transient cerebral ischaemia were studied in the gerbil brain using receptor autoradiography. Transient ischaemia was induced for 10 min. [^{3}H]cAMP binding in the stratum oriens and pyramidale of the hippocampal CA1 sector significantly decreased in the early post-ischaemic stage and showed severe reduction 7 days and 1 month after recirculation. By contrast, [^{3}H]cAMP binding showed no significant alterations in the stratum radiatum of the hippocampal CA1 sector and the stratum pyramidale of the hippocampal CA3 sector up to 48 h after ischaemia. However, the binding in these areas significantly decreased 7 days and 1 month after ischaemia. The stratum lacunosum-moleculare of the hippocampal CA1 sector and dentate gyrus showed no significant changes in [^{3}H]cAMP binding throughout the recirculation period. However, in the dorsolateral part of the striatum, where severe neuronal damage was seen morphologically, [^{3}H]cAMP binding was significantly reduced only one month after ischaemia. These results indicate that marked alteration of intracellular signal transduction precedes neuronal damage in the hippocampal CA1 sector, but not in the striatum. Furthermore, our autoradiographic data suggest that post-ischaemic alteration in [^{3}H]cAMP binding between the hippocampal CA1 sector and striatum may be produced by different mechanisms.

Transient cerebral ischaemia leads to neuronal damage in selectively vulnerable areas. The hippocampal CA1 pyramidal neurons and hilar neurons of dentate gyrus are most vulnerable to brief ischaemia, followed by the striatal neurons, cortical neurons, and thalamic neurons (Pulsinelli et al 1982; Johansen et al 1987; Benveniste & Diemer 1988; Araki et al 1989). Several studies have demonstrated that intracellular second messengers are involved in the pathogenesis of ischaemic brain damage (Jorgensen et al 1989; Onodera et al 1989; Cardell et al 1990) and in neurotransmitter release (Malenka et al 1986; Nishizuka 1986). We have reported that transient ischaemia caused post-ischaemic alteration in the binding sites of protein kinase C, inositol 1,4,5-triphosphate, and forskolin in selectively vulnerable areas (Araki et al 1992a). Furthermore, we have demonstrated that transient ischaemia produced marked reduction in [^{3}H]cAMP binding in the gerbil hippocampus 7 days after recirculation (Araki et al 1992b). Therefore, alterations in binding of cyclic (c) AMP as well as protein kinase C, inositol triphosphate and forskolin are considered to play a vital role in the development of ischaemic neuronal damage. The purpose of the present study was to investigate regional alterations in [^{3}H]cAMP binding after transient ischaemia in the gerbil using receptor autoradiography.

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Materials and methods

Animals and operative procedures. Male Mongolian gerbils (Seiwa Experimental Animals, Fukuoka, Japan), 65–95 g, were anaesthetized with 2% halothane in a mixture of 70% N_2O and 30% O_2. The bilateral common carotid arteries were exposed, anaesthesia was discontinued, and the arteries were clamped with aneurysmal clips for 10 min. The animals adopted a squatting posture without moving their limbs for at least 1 h, enabling the following procedures to be carried out without causing pain. After occlusion, the clips were removed and the gerbils were allowed to survive for 1, 5, 24 and 48 h, 7 days and 1