Biochemical Pharmacology, Vol. 45, No. 10, pp. 1947–1951, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

REOXYGENATION FOLLOWING HYPOXIA STIMULATES LIPID PEROXIDATION AND PHOSPHATIDYLINOSITOL BREAKDOWN IN KIDNEY CORTICAL SLICES

LISA A. COTTERILL, JON D. GOWER, PETER K. CLARK,* BARRY J. FULLER,†
MAUREEN S. THORNILEY,‡ J. GRAHAM GODDARD and COLIN J. GREEN

Section of Surgical Research and *Division of Medical Statistics, Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ; and †Academic Department of Surgery, Royal Free Hospital and School of Medicine, Pond Street, Hampstead, London NW3 2QG, U.K.

(*Received* 16 *November* 1992; *accepted* 5 *March* 1993)

Abstract—Reoxygenation of hypoxic (120 min at 37°) rabbit kidney cortical slices *in vitro* resulted in a rapid increase in lipid peroxidation and phosphatidylinositol hydrolysis. No changes in phosphatidylinositol breakdown occurred during hypoxia or upon reoxygenation in the absence of calcium. Incubation of renal slices with carbon tetrachloride resulted in increased lipid peroxidation but had no effect on phosphatidylinositol breakdown. It is concluded that altered intracellular calcium homeostasis during reoxygenation is involved in mediating increased phosphatidylinositol hydrolysis through activation of a specific phospholipase C, but that oxidative stress *per se* does not have a significant effect on the inositol phosphate secondary messenger response in this model system.

Irreversible damage to cells and organ failure due to ischaemic insult followed by reperfusion represent the end-points of a multitude of adverse biochemical derangements. There is considerable evidence that the over-production of oxygen-derived free radicals and loss of cellular calcium homeostasis are two key features in the pathogenesis of this condition. These two events are linked in a complexity of ways. For instance, increased cytosolic calcium levels due to the failure of energy-dependent pumps under hypoxia activate phospholipases which render membranes and the released free fatty acids more susceptible to oxidative damage [1]. In addition, calcium-dependent proteases can convert xanthine dehydrogenase to the superoxide-generating form xanthine oxidase [2]. Similarly, a high mitochondrial calcium load, which is a consistent feature of reperfusion injury [3], may be associated with increased leakage of reducing equivalents from the electron transport chain onto oxygen. In turn, oxygen radicals inactivate pumps responsible for calcium extrusion from the cell [4] and oxidative damage to intracellular organelles impairs their ability to sequester excess calcium from the cytosol [5].

An important pathway by which cytosolic calcium levels are controlled is through the inositol phosphate (IP§) secondary messenger system [6]. Binding of agonists to plasma membrane receptors results in cleavage of phosphatidylinositol-4,5,-bisphosphate (PIP_2) by a G-protein phospholipase C. This generates two second messenger products, inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 mobilizes calcium from intracellular stores and DAG stimulates phosphorylation and hence activation of protein kinase C, a process which requires phospholipids and calcium for maximum activity [7]. In the kidney, phosphatidylinositol hydrolysis through activation of α_1-adrenoceptors evokes a multiple response: increased sodium reabsorption [8], prostanoid production and vasoconstriction [9], gluconeogenesis [10], and inhibition of renin release [11].

We have previously found evidence for altered intracellular calcium homeostasis in ischaemic rabbit kidneys [12] and a highly elevated rate of lipid peroxidation in ischaemic kidney homogenates [13]. In the present study, we have exposed rabbit kidney cortical slices to hypoxia and reoxygenation *in vitro* and demonstrate that lipid peroxidation and phosphatidylinositol hydrolysis are rapidly stimulated at the time of reoxygenation. In order to gain insight into possible relationships between these two processes we investigated the effect of oxidative stress induced by carbon tetrachloride (CCl_4) on this secondary messenger system.

MATERIALS AND METHODS

Cortical slices were prepared from New Zealand White rabbit kidneys as described previously [14], suspended (25% v/v) in Krebs–Ringer bicarbonate solution (KRB) containing pyruvate (10 mM) and gassed with 95% O_2:5% CO_2 to restore ATP levels. Myo-(2-^{3}H)-inositol (0.32 μm; 19.9 Ci/mmol; Amersham International, Amersham, U.K.) was incorporated into the phospholipids of the slices over a 60 min incubation period at 37°. The slices were then washed four times and resuspended in fresh KRB (25% v/v).

Aliquots of [^{3}H]inositol-loaded slices were sus-

‡ Corresponding author. Tel. (081) 869 3287; FAX (081) 869 3270.

§ Abbreviations: IP, inositol phosphate; IP_1, inositol-1-phosphate; IP_2, inositol-1,4-biphosphate; IP_3, inositol triphosphate; DAG, diacylglycerol; PIP_2, phosphatidylinositol-4,5,-bisphosphate; KRB, Krebs–Ringer buffer; TBA, thiobarbituric acid.

pended (10% v/v; final volume 12 mL) in either KRB buffer (2.5 mM $CaCl_2$), or calcium-free KRB buffer containing 10 mM EGTA, gassed with either 95% O_2:5% CO_2 for 2 min (control) or N_2 for 5 min to create hypoxic conditions and incubated in tightly stoppered flasks for 120 min at 37°. All slices were then gassed with 95% O_2:5% CO_2 and incubated for a further 30 min. Duplicate aliquots (750 μL) were removed at 0, 30, 60, 90 and 120 min and following the final incubation period. Samples were immediately added to chloroform:methanol (2:1 v/v; 3 mL), vortexed for 2 min and the aqueous phase was separated by centrifugation.

Water-soluble IPs were analysed by HPLC (Partisil SAX 10 column; 4.6 mm i.d. × 25 cm; HPLC Technology, Cheshire, U.K.) after spiking with adenine nucleotide markers according to the method of Irvine *et al.* [15]. The eluent was collected in 1.25 mL fractions and the radioactivity of each fraction determined by liquid scintillation counting. Phosphatidylinositol breakdown was also determined by a simple procedure in which IPs were separated on Dowex anion-exchange columns (2 mL of a 50% (w/v) slurry of Dowex-1 resin, 100–200 mesh, X8 in the formate form, BDH Chemicals, Dorset, U.K.) [7]. Inositol and glycerophosphoinositol were eluted with 60 mM ammonium formate/5 mM sodium borate (12 mL) (fraction 1) and inositol phosphates [inositol-1,4-phosphate (IP_1), inositol-1,14,-bis-phosphate (IP_2) and IP_3] with 1 M ammonium formate/0.1 M formic acid (12 mL) (fraction 2). The ratio of the radioactivities in fraction 2: fraction 1 was taken as a measure of IP production.

Lipid peroxidation was determined in duplicate aliquots (1 mL) of the slices at 30 min intervals throughout the incubation period by measuring the formation of thiobarbituric acid (TBA)-reactive material as described previously [13]. This assay was calibrated using malondialdehyde tetraethyl acetal (Sigma Chemical Co. Poole, U.K.). In a separate set of experiments, CCl_4 was added (5 μL; final concentration approximately 5.7 mM) to aliquots (12 mL) of [^{3}H]inositol-labelled slices which were incubated on a rotary shaker for 60 min at 37°. Duplicate samples (1 mL) were taken at 0, 20, 40 and 60 min of the incubation period and IP production and lipid peroxidation determined as described above.

Statistical analysis was performed using analysis of variance (ANOVA) [16]. The residuals from the ANOVA were checked for normal distribution using the Shapiro–Wilks W test [17] and for equal variances

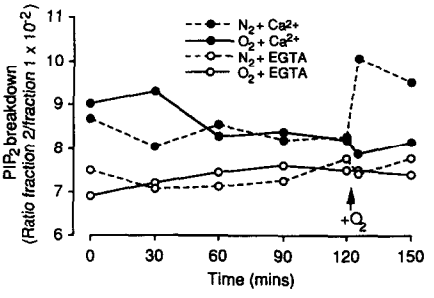

Fig. 2. Formation of IPs in kidney cortical slices incubated at 37° in KRB containing calcium (2.5 mM) (●) or EGTA (10 mM) (○) for 120 min under an atmosphere of N_2 (----) or O_2 (——) followed by reoxygenation and aerobic incubation for a further 30 min. Values represent the means of eight separate determinations performed in duplicate.

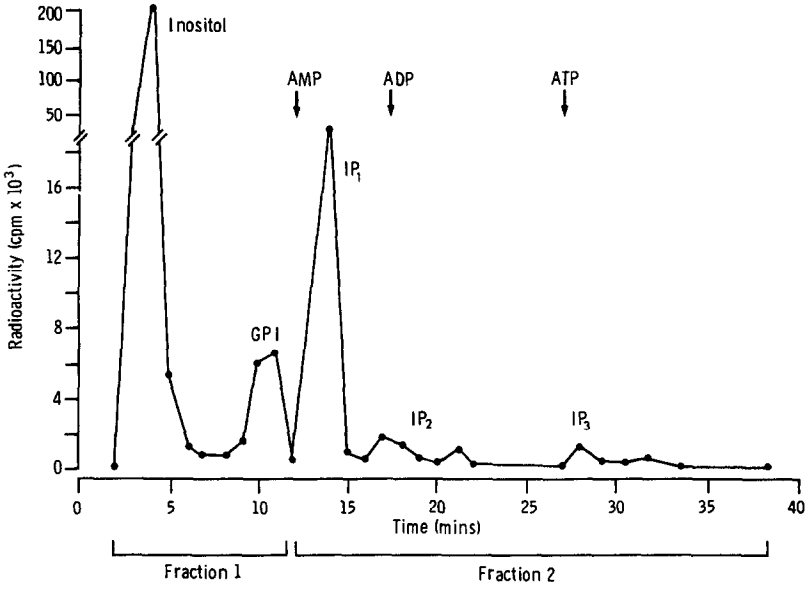

Fig. 1. Analysis by HPLC of IP formation in rabbit kidney cortical slices labelled with [^{3}H]inositol and incubated at 37° for 60 min in KRB.

Table 1. The effect of reoxygenation on IP production in kidney cortical slices following incubation under hypoxic or aerobic conditions in the presence of calcium or EGTA

	$O_2 + Ca^{++}$	$N_2 + Ca^{++}$	O_2 + EGTA	N_2 + EGTA
	Formation of IPs (ratio fraction 2/fraction 1 × 10^{-2})			
Pre-O_2	8.21	8.29	7.51	7.80
Post-O_2	7.97	10.07	7.53	7.45
Change	−0.24	+1.78	+0.02	−0.35
95% CI for change	−1.11, 0.63	0.91, 2.65	−0.85, 0.89	−1.22, 0.52
P-value	0.58	0.002	0.97	0.43

Values are the means and 95% confidence intervals (CI) of eight separate determinations (ratio fraction 2/fraction 1) as described in Materials and Methods and performed in duplicate.

Kidney cortical slices were incubated at 37° in KRB containing calcium (2.5 mM) or EGTA (10 mM) under an atmosphere of N_2 or O_2 for 120 min (pre-O_2) and then regassed with O_2 (post-O_2). P-value refers to pre- versus post- mean ratio value.

in the atmosphere, chemical and time point groups using the Schweder test [18]. When constructing confidence intervals and comparing the means of groups using *t*-tests and the appropriate mean square error from the ANOVA was used as the variance estimate.

RESULTS

Effect of hypoxia/reoxygenation

Incubation of [^{3}H]inositol-labelled kidney cortical slices resulted in the formation of radiolabelled IP_1 and smaller amounts of IP_2 and IP_3 (Fig. 1). IP_3 is produced from the cleavage of PIP_2 and is sequentially metabolized to IP_2 and IP_1 by phosphatases. IP_2 and IP_1 may also be produced by phospholipase C action on the lipid precursors of PIP_2, namely phosphatidylinositol-4-phosphate and phosphatidylinositol itself.

There was no change in the production of IPs as measured by Dowex chromatography in the hypoxic slices during the 120 min incubation period in an atmosphere of N_2 compared with control slices incubated under aerobic conditions either in the presence of calcium or in calcium-free medium containing EGTA (Fig. 2). However, immediately upon reoxygenation of the hypoxic slices incubated in the presence of calcium, IP production increased rapidly (Fig. 2). Statistical analysis of the results (Table 1) showed that this increase was highly significant ($P = 0.002$) but in marked contrast reoxygenation of hypoxic slices in calcium-free medium (+EGTA) had no effect on the rate of IP production. Furthermore, no significant changes in IP production were measured in slices incubated in the presence of O_2 and EGTA (Table 1 and Fig. 2) or in the presence of calcium and oxygen upon oxygenation with 95% O_2: 5% CO_2 (Table 1 and Fig. 2).

The formation of lipid peroxidation products increased during the first 60 min of incubation in the presence of O_2 and then levelled off over the remaining period (Fig. 3). Lipid peroxidation was also evident in the hypoxic slices but to a lesser

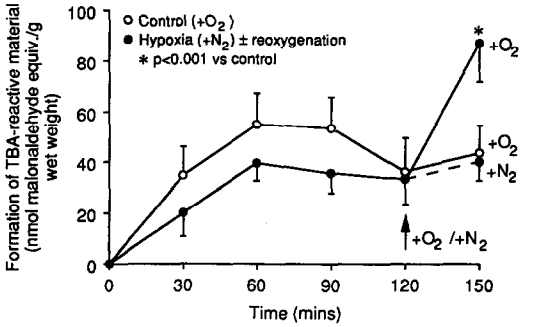

Fig. 3. Lipid peroxidation in kidney cortical slices incubated at 37° in KRB for 120 min under an atmosphere of N_2 (●) or O_2 (○) followed by regassing with either O_2 (——) or N_2 (– – –) and incubation for a further 30 min. Values are the means ± 95% confidence limits of six separate determinations performed in duplicate.

degree than in the oxygenated samples comparing overall means of control vs hypoxic determinations over 120 ($P = 0.002$) min (Fig. 3). Reoxygenation following 120 min of hypoxia resulted in a significant ($P < 0.001$) increase in the rate of lipid peroxidation compared with either the slices incubated under oxygen or those kept continuously under N_2 (Fig. 3).

The effect of carbon tetrachloride

In order to investigate the effect of oxidative stress on IP production, rabbit kidney cortical slices were incubated with CCl_4. This agent is metabolized by the cytochrome P450 system to the trichloromethyl radical ($CCL_3^{\cdot}$) which then reacts with molecular oxygen to form the more reactive trichloromethyl peroxy radical ($CCl_3O_2^{\cdot}$), the likely candidate for initiating lipid peroxidation [19].

Incubation of slices in the presence of carbon tetrachloride resulted in an elevated rate of peroxidation, the amount of TBA-reactive material being significantly greater than controls at 40 min

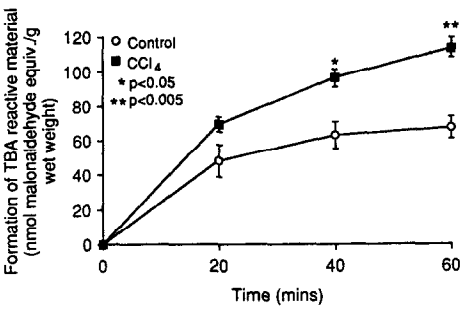

Fig. 4. Lipid peroxidation in kidney cortical slices incubated at 37° in KRB in the presence or absence of CCl_4 (5.7 mM). Values are the means ± 95% confidence limits of six separate determinations performed in duplicate.

($P < 0.05$) and 60 min ($P < 0.005$) (Fig. 4). These results showed that CCl_4 was an effective initiator of lipid peroxidation in this model system.

The effect of CCl_4 on IP production was determined by incubating cortical slices in either KRB or calcium-free KRB (containing EGTA). Ca^{2+} was required to effect an increase in IP turnover over a 60 min aerobic incubation period, mean ratio $(7.15 \pm 1.46\ SD) \times 10^{-2}$ compared to mean $(5.91 \pm 1.26) \times 10^{-2}$ in the presence of EGTA. However, the addition of CCL_4 had no effect on IP production in the presence of calcium, mean $(7.86 \pm 1.38) \times 10^{-2}$ compared to the ratio measured in the presence of calcium alone, mean $(7.15 \pm 1.46) \times 10^{-2}$.

DISCUSSION

Reoxygenation of hypoxic cortical slices resulted in an elevated rate of lipid peroxidation which is in agreement with our previous studies on whole rabbit kidneys rendered ischaemic *in vivo* [13]. This is likely to be due to several different events occurring during both hypoxia and reoxygenation including: redistribution of intracellular iron [20], which is involved in the initiation of lipid peroxidation; altered intracellular calcium homeostasis, which activates phospholipase A_2 and renders membranes more susceptible to free radical attack [1], and increased production of oxygen-derived free radicals due to mitochondrial dysfunction and activation of xanthine oxidase [2].

The present results demonstrate that hydrolysis of phosphatidylinositols to secondary messenger products is activated very rapidly upon reoxygenation of renal tissue following hypoxia but that no change in IP production occurs during the hypoxic period itself. Production of IP_3 which is solely derived from PIP_2 was demonstrated by HPLC, whilst anion exchange chromatography was used for most of the experiments to assess rapidly total IP formation in reoxygenated slices. Increased turnover of phosphatidylinositols upon reperfusion has also been demonstrated previously in isolated ischaemic rat hearts [21] but the exact nature of the IPs was not determined. In synaptosomal fractions, stimulation of phospholipase C activity on phosphatidylinositol of PIP_2 hydrolysis was suppressed in ischaemic brain tissue [21, 22].

The stimulation of phosphatidylinositol breakdown on reoxygenation may have been due to increased oxidative stress, which is exemplified in this model system by the increase in lipid peroxidation. One of the products of lipid peroxidation, 4-hydroxynonenal, has been shown to stimulate adenylate cyclase, guanylate cyclase and PIP_2 breakdown *in vitro* [23]. In addition, lipid peroxidation alters membrane configuration [24], which may affect the interaction of phospholipase C with membrane-bound regulatory components or PIP_2, thus making its substrate more accessible. The possibility that increased rates of lipid peroxidation may stimulate phosphatidylinositol hydrolysis was investigated by initiating this process in renal cortical slices with CCL_4. This agent proved effective at stimulating lipid peroxidation and, because of the requirement for metabolic activation of CCL_4, it could be concluded that lipid peroxidation was taking place intracellularly. The level of TBA-reactive material following 40–60 min incubation of slices with CCl_4 was very similar to that found upon reoxygenation of slices rendered hypoxic. However, despite a steady increase in IP formation in slices exposed to CCl_4 for 20–60 min which followed the increase in lipid peroxidation over this time period, no statistically significant changes in IP formation could be demonstrated. Thus, it can be concluded that lipid peroxidation does not itself dramatically affect the rate of IP production under these conditions.

It is possible that the increased IP production upon reoxygenation was the result of altered intracellular calcium homeostasis as the phospholipase C responsible for cleavage of phosphatidylinositols is metal ion dependent. During ischaemia, cytosolic calcium levels, which are normally maintained at very low levels (10^{-7} M) compared with the extracellular fluid (10^{-3} M), may increase due to failure of energy-dependent pumps, which export calcium from the cell or sequester it in intracellular organelles. However, perturbations in cell calcium due to hypoxia itself were insufficient to evoke a response in phosphatidylinositol cleavage during this period. On reoxygenation much more dramatic changes to calcium homeostasis can occur due to loss of calcium-sequestering ability of damaged intracellular organelles and increased permeability of the plasma membrane allowing entry of extracellular calcium. In the presence of EGTA, no stimulation of IP formation was observed upon reoxygenation which supported a role for calcium in mediating this post-hypoxic response.

The physiological consequences of rapid hydrolysis of phosphatidylinositols on reoxygenation following ischaemia have yet to be assessed. Enhanced production of IP_3 may contribute to increases in cytosolic calcium levels with subsequent activation of a range of calcium-dependent enzymes including phospholipase A_2. Deregulation of receptor-mediated function through this intracellular secondary messenger system may cause imbalances in eicosanoid production in the endothelium through the DAG-mediated activation of protein kinase C.

The resulting vascular disturbances may subsequently contribute to the pathogenesis of post-ischaemic reperfusion damage to transplanted kidneys.

Acknowledgements—This research was supported by The Medical Research Council and the Restoration of Appearance and Function Trust.

REFERENCES

1. Cotterill LA, Gower JD, Fuller BJ and Green CJ, Free fatty acid accumulation following cold ischaemia in rabbit kidneys and the involvement of a calcium-dependent phospholipase A2. *Cryolett* **11**: 3–12, 1990.
2. Roy RS and McCord JM, Superoxide and ischemia: conversion of xanthine dehydrogenase to xanthine oxidase. In: *Oxy Radicals and their Scavenger Systems* (Eds. Greenwald R and Cohen G), Vol. 2, pp. 145–153. Elsevier Science, New York, 1983.
3. Arnold PE, Lumlertgul D, Burke TJ and Schrier RW, *In vitro* versus *in vivo* mitochondrial calcium loading in ischemic acute renal failure. *Am J Physiol* **248**: 845–850, 1985.
4. Nicotera PL, Moore M, Mirabelli F, Bellomo G and Orrenius S, Inhibition of hepatocyte plasma membrane Ca^{2+} ATPase activity by menadione metabolism and its restoration by thiols. *FEBS Lett* **181**: 149–153, 1985.
5. Bellomo G, Richelmi P, Mirabelli F, Marinoni V and Abbagnano A, Inhibition of liver microsomal calcium ion sequestration by oxidative stress: role of protein sulphydryl groups. In: *Free Radicals in Liver Injury* (Eds. Poli G, Cheeseman KH, Dianzani MU and Slater TF), pp. 139–142. IRL Press, Oxford, 1985.
6. Berridge MJ, Inositol triphosphate and diacylglycerol as secondary messengers. *Biochem J* **220**: 354–360, 1984.
7. Berridge MJ, Dawson RMC, Downes CP, Heslop JP and Irvine RF, Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. *Biochem J* **212**: 473–482, 1983.
8. Hesse IFA and Johns EJ, The subtype of α-adrenoceptor involved in the neural control of renal tubular sodium reabsorption in the rabbit. *J Physiol* **328**: 527–538, 1984.
9. Cooper CL and Malik KU, Prostaglandin synthesis and renal vasoconstriction elicited by adrenergic stimuli are linked to activation of alpha-1 adrenergic receptors in the isolated rat kidney. *J Pharmacol Exp Ther* **233**: 24–31, 1985.
10. Kessar P and Saggerson ED, Evidence that catecholamines stimulate renal gluconeogenesis through an α_1-type of adrenoceptor. *Biochem J* **190**: 119–123, 1980.
11. Matsumura Y, Miyawaki N, Sasaki Y and Morimoto S, Inhibitory effects of norepinephrine, methoxamine and phenylephrine on renin release from rat kidney cortical slices. *J Pharmacol Exp Ther* **233**: 782–787, 1985.
12. Cotterill LA, Gower JD, Fuller BJ and Green CJ, Oxidative damage to kidney membranes during cold ischaemia: evidence of a role for calcium. *Transplantation* **48**: 745–751, 1989.
13. Green CJ, Healing G, Lunec J, Fuller BJ and Simpkin S, Evidence of free-radical-induced damage in rabbit kidneys after simple hypothermic preservation and autotransplantation. *Transplantation* **41**: 161–165, 1986.
14. Fuller BJ, Marley SPE and Green CJ, Glucuneogenesis in stored kidneys from normal and cold-acclimated rats. *Cryolett* **6**: 91–98, 1985.
15. Irvine RF, Anggard EE, Letcher AJ and Downes CP, Metabolism of inositol 1,4,5-trisphosphate and inositol 1,3,4-trisphosphate in rat parotid glands. *Biochem J* **229**: 505–511, 1985.
16. Armitage P and Berry G, *Statistical Methods in Medical Research*. Blackwell Scientific, Oxford, 1987.
17. Royston JP, A simple method for evaluating the Shapiro–Francia W test of non-Normality. *The Statistician* **32**: 297–300, 1983.
18. Schweder T, A simple test for a set of sums of squares. *Appl Stat* **30**: 16–21, 1981.
19. Packer JE, Slater TF and Willson RL, Reaction of the carbon tetrachloride related peroxy free radical ($CCl_3O_2^{\cdot}$) with amino acids: pulse radiolysis evidence. *Life Sci* **23**: 2617–2620, 1987.
20. Gower JD, Healing G and Green CJ, Measurement by hplc of desferrioxamine-available iron in rabbit kidneys to assess the effect of ischaemia on the distribution of iron within the total pool. *Free Rad Res Commun* **5**: 291–299, 1989.
21. Otani H, Prasad MR, Engleman RM, Otani H, Cordis GA and Das DK, Enhanced phosphodiesteratic breakdown and turnover of phosphoinositides during reperfusion of ischemic rat heart. *Circ Res* **63**: 930–936, 1988.
22. Strosznajder J, Prolonged ischemia differently affects phospholipase C acting against phosphatidylinositol and phosphatidylinositol 4,5-bisphosphate in brain subsynaptosomal fraction. *FEBS Lett* **257**: 110–112, 1989.
23. Dianzani MU, Paradisis L, Barrera G, Rossi MA and Parola M, The action of 4-hydroxynonenal on the plasma membrane enzymes from rat hepatocytes. In: *Free Radicals, Metal Ions and Biopolymers* (Eds. Beaumont PC, Deeble DJ, Parsons BJ and Rice-Evans C), pp. 329–346. Richelieu Press, London, 1989.
24. Ungemach FR, Plasma membrane damage of hepatocytes following lipid peroxidation: involvement of phospholipase A2. In: *Free Radicals in Liver Injury* (Eds. Poli G, Cheeseman KH, Dianzani MU and Slater TF), pp. 127–134. IRL Press, Oxford, 1985.