



INDUCTION OF IMMEDIATE-EARLY, ORNITHINE DECARBOXYLASE AND ANTIZYME GENE EXPRESSION IN THE RAT SMALL INTESTINE AFTER TRANSIENT ISCHAEMIA

Z. Pujic¹, I. Matsumoto, A. Yamataka*, T. Miyano* and P. Wilce

Department of Biochemistry, The University of Queensland
St Lucia, Queensland, 4072, Australia.

*Department of Paediatric Surgery, Juntendo University School of Medicine,
Tokyo, Japan.

(Received in final form April 17, 1996)

Summary

The expression of the immediate early genes (IEG)s *c-fos*, *c-jun* and *zif/268*, and the genes coding for ornithine decarboxylase (ODC) and its regulatory protein antizyme (AZ), was studied in rat small intestine following transient ischemia. The ischemic stimulus for 10 min alone did not alter the expression of these genes. A rapid and transitory induction of all IEG mRNAs occurred in a coordinated manner peaking at 30 min following recirculation and returned to basal levels 3 hr after recirculation. Protein products of the IEGs accumulated in the smooth muscle layer of the intestine by 2-3 hr after recirculation. Expression of both ODC and AZ mRNAs initially decreased to 70% of control levels 1 hr after recirculation but markedly increased at 2 to 4 hr after recirculation. The functional significance of these changes in gene expression in relation to tissue integrity and function after the ischaemia/reperfusion is discussed.

Key Words: immediate early genes, ornithine decarboxylase, ischaemia, intestine

Small bowel transplantation (SBT) has become a feasible clinical procedure (1), but it is not yet as widely acceptable as other organ transplantation due to the difficulty of organ procurement and preservation. Small intestinal grafts are easily damaged by warm ischaemia, cold preservation, rewarming and reperfusion during the transplantation process (2). Ischemic tissue damage has been associated with free radical formation, however the subsequent gene expression that may initiate repair or protective measures to prevent further damage have yet to be elucidated. A molecular analysis of the gene expression that may trigger the ischemically damaged small intestine to initiate recovery or further damage may allow a more precise elucidation of the mechanism of SBT-related ischaemia/reperfusion injury and may lead to a new approach to improve organ procurement in SBT.

¹To whom all correspondence should be addressed

The molecular and biochemical events that accompany ischaemia/reperfusion in the small intestine are poorly understood. Immediate-early genes (IEG)s are rapidly and transiently expressed after a wide variety of chemical and physical stimuli in almost all tissues studied (3,4,5). Several IEGs code for proteins which can associate via a characteristic leucine zipper motif to form functional transcription factors (6,7). FOS and JUN heterodimers and JUN homodimers are capable of modulating transcription of target genes by binding to specific DNA regulatory elements (AP-1 binding sites) (6). It is suggested that these gene products function as a link between the extracellular signals and long-term changes in gene expression (8). Another IEG, *zif/268* codes for a protein with a zinc-finger motif which interacts with a different but characteristic DNA sequence to influence other target genes (9). Following transient ischaemia of the brain, liver and kidney (10,11), transcription of the IEGs, *c-fos* and *c-jun* occurs rapidly after recirculation. The expression of IEGs after a given stimulus, such as ischaemia and reperfusion may play an important role in the modification of tissue integrity and function.

Ornithine decarboxylase (ODC) plays a key role in the regulation of polyamine synthesis in many tissues (12). An important regulator of ODC activity is the polyamine-inducible protein antizyme (AZ) (13). Recent work (14) has shown that the AZ/ODC complex binds to the 26S proteasome resulting in proteolysis of ODC protein in an ATP/AZ, ubiquitin-independent manner. Polyamines have been implicated in proliferation of many cell types and in tissue repair (15,16,17,18). In rat intestine, inhibition of ODC activity arrests mucosal recovery following damage and retards intestinal development in the neonate (19). This suggests that ODC activity, and the consequent changes in tissue polyamine levels, play an important role in tissue repair.

In the present study, as a first step in evaluating the molecular mechanisms of ischaemia-reperfusion injury in the small intestine, we investigated the expression of *c-fos*, *c-jun* and *zif/268* as well as ODC and AZ after the transient ischaemia/reperfusion stimulus.

Methods

1. Treatment of Animals

Male Wistar rats, weighing 200 to 300 g, were anaesthetised with an intraperitoneal injection of pentobarbital (40 mg/kg) and anaesthesia was maintained for 4 hours by repeated injections. The superior mesenteric artery was clamped for 10 min with microarterial clamps. Recirculation was initiated at zero time and sections of the jejunum (2 cm) were removed immediately after reperfusion. Further sections were removed at 30 min, and 1, 2, 3 and 4 hr thereafter. Tissue was pooled from two animals. Sham-operated animals were anaesthetised, the artery was touched but not clamped for 10 min and sections of intestine were removed as above. The tissue was cut longitudinally, quickly rinsed in physiological saline at 4°C and immediately frozen in liquid nitrogen before storage at -70°C until use. Procedures were in accordance to those approved by the Animal Experimental Ethics Committee of The University of Queensland.

2. Isolation and Analysis of RNA

Total jejunum RNA was isolated by the guanidinium-thiocyanate method as previously described (20) and separated using a 1% w/v agarose, 6.6% v/v formaldehyde gel. Integrity of the RNA was assessed by staining the RNA with 0.5 µg/mL ethidium bromide. The RNA was then capillary transferred onto Hybond-N membrane (Amersham, Australia) using 10 X SSC (1X SSC is 0.15 M sodium citrate, 15 mM NaCl) and UV-crosslinked. The membranes were prehybridised for 15 min at 65°C in Rapid Hybridisation Solution (Amersham, Australia). The cDNA probes were labelled

with ^{32}P -dCTP by random priming using the Pharmacia QuickPrime procedure and added directly to the prehybridisation solution. After hybridisation at the same temperature for 2 hr, the membranes were washed at a final stringency of 65°C in 0.1X SSPE (1X SSPE is 0.15 M NaCl, 10 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 1 mM EDTA), 0.1% w/v SDS at 65°C for 30 min and exposed to Kodak XAR-5 film with intensifying screens at -70°C for a few days. Probes were removed from membranes by washing in 1% SDS at 95°C for 10 min and blots were rehybridised with a ^{32}P -labelled oligonucleotide probe specific for the 18S rRNA to correct for variation in the loading and transfer of RNA.

3. Immunohistochemistry

The immunohistochemical procedure to detect the IEG protein products was carried out as previously described (20). Briefly, animals were anaesthetised and perfused intracardially with 4% (w/v) paraformaldehyde. 2 cm sections of the jejunum were excised, postfixed in 4% paraformaldehyde overnight, cryoprotected in 30% sucrose in PBS (w/v) and embedded in OCT prior to cryostat sectioning. Sections ($48\ \mu\text{m}$) were washed in PBST, incubated in 50% ethanol and then washed in 50% (v/v) ethanol with 2% H_2O_2 (v/v) in 50% ethanol to remove endogenous peroxidase activity. After washing with PBST, the tissue was incubated with polyclonal anti-rabbit primary antibodies (anti-C-FOS, 1:10000, anti-C-JUN, 1:10000, anti-ZIF/268, 1:5000) (21) for 48 hr at 4°C . After washing in PBST, the tissue was incubated in biotinylated secondary antibodies (Vectastain, Vector Laboratories) for 2 hr and the ABC complex (Vectastain, Vector Laboratories) was applied for 1 hr. After staining with nickel enhanced diaminobenzidine, the tissue was mounted onto glass slides, allowed to air dry and then coverslipped for examination by light microscopy.

4. Probes

DNA probes used were: *c-fos* obtained from T. Curran, Roche Centre NJ, U.S.A., *c-jun* (pcD10) obtained from I. M. Verma, Salk Institute, La Jolla, U.S.A., *zif/268* (268BS65) obtained from D. Nathans, Johns Hopkins University School of Medicine, Baltimore, USA, ODC obtained from, P. Coffino, University of California, San Francisco, USA and AZ (pT7NAZ) was obtained from S. Hayashi, Jikei Medical School, Tokyo, Japan.

Results

The ischaemic stimulus alone failed to induce any IEG expression (Fig. 1). In contrast, there was a marked increase of *c-fos*, *c-jun* and *zif/268* expression which peaked at 30 min after recirculation (Fig. 1). Levels of *c-jun* mRNA 30 min after recirculation were approximately 2-fold greater than in the zero time sample, while 12- and 20-fold increases of *c-fos* and *zif/268* were observed 30 min after reperfusion respectively. The levels of all IEG mRNAs returned to basal levels by 3 h. An autoradiogram representing IEG expression at 30 min after ischaemia/reperfusion compared to sham-operated animals is shown in Figure 2. We were unable to detect any changes in IEG expression at any time in the tissue of sham-operated animals. The timing of these changes in IEG expression is very similar to mucosal permeability changes observed in the same model (22) and suggests that the IEG induction is mediated by a factor present in the intestinal lumen.

ODC is the rate limiting enzyme in polyamine synthesis. The induction of ODC mRNA and of ODC enzyme activity, and the rise of polyamine levels have been suggested to be important events in gut repair (23). We therefore monitored the expression of ODC in intestinal tissue after transient ischaemia (Fig. 3). Relatively high levels of the 2.0 and 2.4 kb ODC transcripts were observed in the unstimulated intestine. Ischaemia alone did not alter the level of this expression.

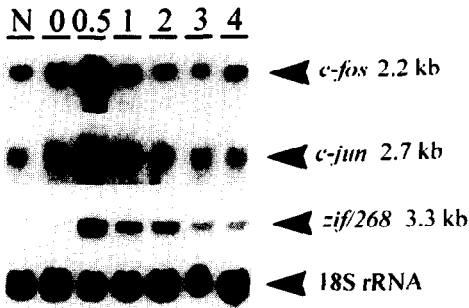


Fig. 1

Expression of *c-fos* (black), *c-jun* (hatched) and *zif/268* (white) between 0 and 4 hr following 10 min of ischemia. N represents the signal from a naive control. Data are means of two separate experiments normalised with respect to 18S rRNA. A representative northern is shown.

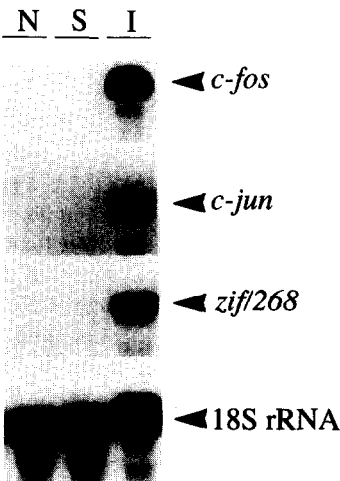
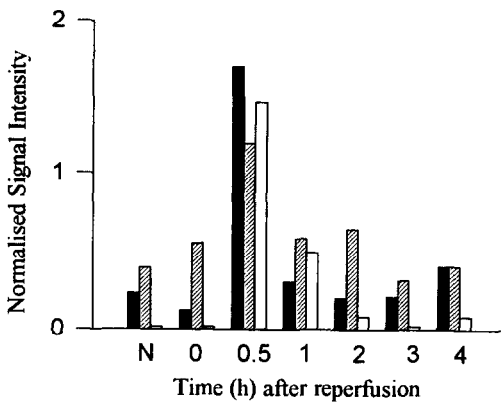


Fig. 2

Expression of *c-fos*, *c-jun* and *zif/268* in ischemic (I) and sham-treated controls (S) 30 minutes after reperfusion. N represents a naive control sample.

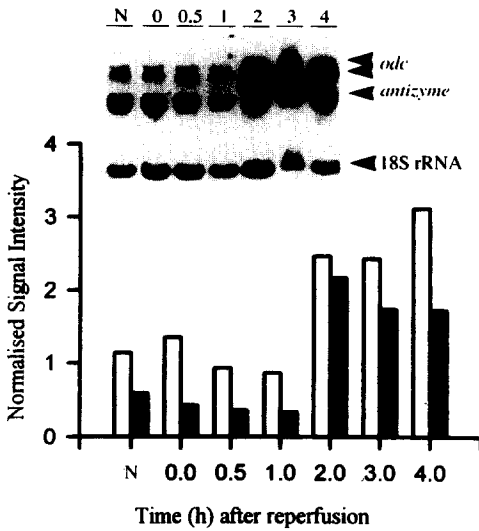


Fig. 3
Expression of ODC (black) and AZ (white) between 0 and 4 hr following 10 min of ischemia. N represents a naive control. Signals are averages of two samples whose optical density was normalised with respect to an 18S rRNA signal. A representative northern is shown.

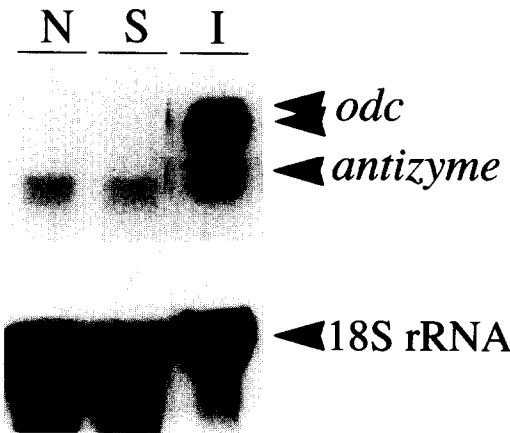


Fig. 4
Expression of ODC and AZ in ischemic (I) and sham-treated controls (S). N represents a naive control.

(Fig. 3). Sham-operations did not alter expression of ODC and AZ mRNAs 3 hr after manipulation (Fig. 4).

In order to identify the cells that are responding to ischaemia/reperfusion with the increased IEG expression, nuclear IEG immunoreactivity (IR) was mapped using specific antibodies. To examine the temporal and spatial expression of the IEG protein products after ischaemia/reperfusion we sampled tissue up to 4 h after reperfusion. There was a low basal IR for any of the IEG products in the muscle layers (Fig. 5). In contrast, high basal IR in the mucosal layer particularly at the tips of the villi was observed (data not shown). An increase in protein products of all three IEGs, *c-fos*, *c-jun* and *zif/268* was observed in the cellular nuclei of the muscle layers peaking at 2 to 3 hr. Representative photomicrographs of tissue 3 hr after reperfusion are shown in Figure 5. The high basal expression of IEG-IR precluded an assessment of the effect of ischaemia/reperfusion on IEG-IR in the mucosal layer.

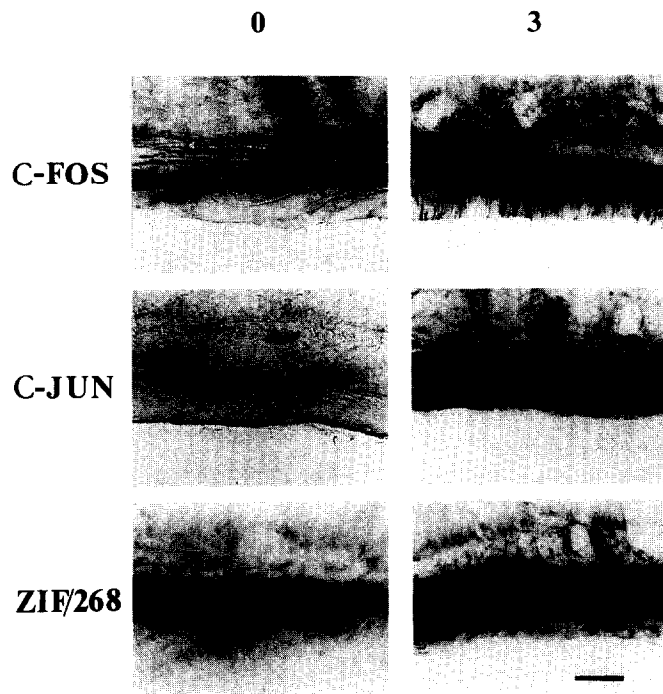


Fig. 5

Immunohistochemical staining of c-FOS, c-JUN and ZIF/268 in jejunum muscle layers at 0 hrs and 3 hrs after reperfusion. Representative fields from three animals. Scale bar = 44 μ m.

Discussion

The present study indicates that 10 min of an ischaemic stimulus followed by reperfusion results in a striking pattern of alteration in the expression of two different categories of genes in the small intestine. We observed very early increases in the expression of the IEGs (*c-fos*, *c-jun* and *zif/268*) which occurred within the smooth muscle compartment. At later time points, coordinated increases occurred in the expression of ODC and AZ. Thus we have demonstrated a complex and temporally related pattern of gene expression within the small intestine upon transient ischaemia/reperfusion.

Accumulated evidence has indicated that the ischaemia/reperfusion stimulus results in induction of IEGs in various organs. In the intestine, coordinated induction of IEGs in mucosal scrapings after starvation and refeeding has been reported (24). Our data presents the first evidence of IEG induction in reperfused small intestinal tissue after transient ischaemia.

Basal levels of c-JUN IR are relatively high in the tissue studied suggesting a possible physiological role for this protein in the maintenance of tissue integrity and function. This maintenance role may be achieved by formation of JUN homodimers or heterodimers with other members of the JUN family or members of the FOS family other than c-FOS. The marked increase in C-FOS, JUN and ZIF/268 expression 3 hr after ischemia/reperfusion may indicate a marked change in tissue gene

expression and the induction of an adaptive response to the ischaemia/reperfusion stimulus. ZIF/268 is a classic zinc finger type of protein (25) presumably influences a different set of LRGs than those activated by FOS or JUN. The IEG expression and transcription factor activation may lead to activation of a number of LRG whose identity remains to be determined.

Although expression of IEGs is transient in most situations, recent evidence indicates that the continuous expression of IEGs appear to predict cell mortality (26). Recent observations from our laboratory suggested that a continuous pattern of IEG expression in various organs at the early stage of ischaemia/reperfusion is well correlated with the severity of organ damage, survival and in the case of transplantation, allograft failure (11). As there is considerable information available concerning the intracellular signalling pathways that regulate IEG expression (27), it may be possible to use these genes as molecular indicators to dissect the signal transduction processes involved in the earliest stage of cell death to assess the organ condition and to predict graft prognosis, in the case of transplantation. This point became significant because there is no reliable and specific predictor for primary graft non-function which occurs at an unacceptable rate in transplantation. Confirmation of IEG induction after warm ischaemia in the intestine encourage us to investigate IEG induction in the intestine after cold ischaemia/reperfusion (SBT) in relation to graft condition.

ODC is the first and rate-limiting enzyme in polyamine biosynthesis (17). Changes in the rate of transcription or translation and stability of mRNA and protein have all been implicated in the modulation of ODC catalytic activity (14, 28).

ODC activity in the mucosa of the rat small intestine is reported to increase after ischaemia/reperfusion (29). Our data indicates that this increase of enzyme activity may be, at least partially, regulated at the transcriptional level. The induction of ODC mRNA and of ODC enzyme activity and the subsequent rise of polyamine levels have been suggested to be important events in gut repair (23). One can speculate that the rise in IEG product leads to accumulation of ODC and AZ mRNA 2 hr after recirculation by the activation of gene expression. The *odc* gene is known to possess sequences capable of binding the AP-1 complex in its 5' region, while an AP-1 binding site regulating the transcription of the AZ gene has not been shown. Alternatively, considering the spatial expression of IEGs, it is also possible that these increases are a separate events, taking place within different compartments. A possible interaction of IEGs on transcriptional control of ODC and AZ awaits further investigation.

References

1. D. GRANT, W. WALL, R. MIMÉAULT, R. ZHONG, G. GHENT, R. GARCIA, C. STILLER and J. DUFF, *Lancet* **335** 181-184 (1990).
2. E. SCHOLTEN, S. MANEK, J. PEARSON and C.J. GREEN, *Trans. Proc.* **24** 1096-1097 (1992).
3. M. DRAGUNOW, D. YOUNG, P. HUGHES, G. MACGIBBON, P. LAWLLOW, K. SINGLETON, E. SIRAMANNE, E. BEILHARZ and P. GLUCKMAN, *Mol. Brain Res.* **18** 347-352 (1993).
4. G. BING, E.A. STONE, Y. ZHANG and D. FILER, *Brain Res.* **592** 57-62 (1992).
5. T. REN and S.M. SAGAR, *Brain Res. Bull.* **29** 589-597 (1992).
6. T. CURRAN and B.R. FRANZA JR, *Cell* **55** 395-397 (1988).
7. R.B. FRANZA JR, F.J. RAUSCHER III, S.F. JOSEPHS and T. CURRAN, *Science* **239** 1150-1153 (1988).
8. W.C. ABRAHAM, M. DRAGUNOW and W.P. TATE, *Mol. Neurobiol.* **5** 297-314 (1991).

9. B. CHRISTY and D. NATHANS, *Proc. Natl. Acad. Sci. U.S.A.* **86** 8737-8741 (1989).
10. W.F. MARTERRE JR, M.S. KINDY, J.M. CARNEY, R.W. LANDRUM and W.E. STODEL, *Surgery* **110** 184-191 (1991).
11. S. GOTO, I. MATSUMOTO, N. KAMADA, A. BUI, T. SAITO, M. FINDLAY, Z. PUJIC and P. WILCE, *Transplantation* **58** 840-845 (1994).
12. B.R. DAS and M.S. KANUNGO, *Exp. Gerontol.* **17** 95-103 (1984).
13. P. LAITINEN, R. HUHTINEN, O. HIETALA and A. PAJUNEN, *J. Neurochem.* **44** 1885-1891 (1985).
14. Y. MURAKAMI, S. MATSUFUJI, T. KAMEJI, S. HAYASHI, K. IGARASHI, T. TAMURA, K. TANAKA and A. ICHIHARA, *Nature* **360** 597-599 (1992).
15. E.R. SEIDEL, M.K. HADDOX and L.R. JOHNSON, *Am. J. Physiol.* **246** G649-G653 (1984).
16. G.D. LUK and S.B. BAYLIN, *Am. J. Physiol.* **245** G656-G660 (1983).
17. A.E. PEGG and H.G. WILLIAMS-ASHMAN, *Biochem. J.* **108** 533-539 (1968).
18. D.H. RUSSEL, *Drug Metab. Rev.* **16** 1-88 (1985).
19. G.D. LUK, L.J. MARTON and S.B. BAYLIN, *Science* **210** 195-198 (1980).
20. I. MATSUMOTO, J. LEAH, B. SHANLEY and P. WILCE, *Mol. Cell. Neurosci.* **4** 485-491 (1993).
21. K. KOVARY and R. BRAVO, *Mol. Cell. Biol.* **11** 2451-2459 (1991).
22. J.C. LANGER, SOHAL, S.S. and R.H. RIDDELL, *J. Pediatr. Surg.* **28** 601-605 (1993).
23. D.H. CHUNG, B.M. EVERS and C.M. TOWNSEND, *Am. J. Surg.* **163** 157-162 (1992).
24. R.A. HODIN, J.R. GRAHAM, S. MENG and M.P. UPTON, *Am. J. Physiol.* **266** G38-G89 (1994).
25. P. LEMAIRE, O. REVELANT, R. BRAVO and P. CHARNAY, *Proc. Natl. Acad. Sci. U.S.A.* **85** 4691-4695 (1988).
26. R.J. SMEYNE, M. VENDRELL, M. HAYWARD, S.J. BAKER, G.G. MIAO, K. SCHILLING, L.M. ROBERTSON, T. CURRAN and J.I. MORGAN, *Nature* **363** 166-169 (1993).
27. J.I. MORGAN and T. CURRAN, *Ann. Rev. Neurosci.* **14** 421-451 (1991).
28. N.H. ZAWIA and G.J. HARRY, *Dev. Brain Res.* **71** 53-57 (1993).
29. K. FUJIMOTO, D.N. GRANGER, V.H. PRICE and P. TSO, *Am. J. Physiol.* **261** G523-G529 (1991).