The intrinsic and extrinsic apoptotic pathways are independently activated in response to oxidative stress in pancreatic acinar cells

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Oxidative stress plays an important role in the pathogenesis of acute pancreatitis. Increased generation of reactive oxygen species is a well known initiator of apoptosis and has been shown to induce apoptosis in mouse pancreatic acinar cells [1]. Opening of the permeability transition pore, release of cytochrome c, and activation of caspase-9 occurs in these cells in response to oxidative stress; however, a small population of cells can still undergo apoptosis when the opening of the permeability transition pore is blocked. We investigated the possible role and mechanism of action of the alternative apoptotic pathways versus the intrinsic pathway in pancreatic acinar cells exposed to oxidant stress.

Mouse pancreatic acinar cells (freshly isolated from CD1 mice) were loaded with a fluorescent substrate for caspase-9 or caspase-8. Using confocal microscopy, fluorescence of cleaved substrates was imaged in real time in response to the oxidant menadione (30 µM). Cells were positive for caspase activation when fluorescence was higher than the average fluorescence of control cells plus two standard deviations. Caspase-9 or caspase-8 activity was examined in control cells and cells pre-treated with 25 µM BAPTA-AM to prevent cytoplasmic calcium elevations, 200 µM TPEN to reduce calcium in the endoplasmic reticulum, 50 µM bongkrekic acid to block opening of the mitochondrial permeability transition pore, 10-40 µM caspase-9 inhibitor, or 50 µM GPN to disrupt lysosomes. An unpaired, two-tailed Student’s t test was used for statistical comparisons between treatment groups (p<0.05 was significant).

Caspase-9 was activated within a few minutes (t1/2 = 129 ± 43 s; n=12) after administration of menadione. Activation of caspase-9 was significantly inhibited in cells pre-treated with BAPTA-AM, but no change was observed in the presence of 200 µM of TPEN (decreased ER calcium content as measured with MagFluo4). Caspase-8 was activated (t1/2) within 26 ± 3 min (n=8) after treatment with menadione in 21 ± 3% of cells (n=716). Activation of caspase-8 was not altered by inhibition of the intrinsic apoptotic pathway with bongkrekic acid or caspase-9 inhibitor. Caspase-8 activation was also not changed in the presence of BAPTA-AM. However, caspase-8 activation was significantly reduced when lysosomes of the cells were destroyed with GPN. Both, the intrinsic and in some cells extrinsic apoptotic pathways are rapidly activated in response to oxidative stress in the pancreatic acinar cell. Caspase-9 activation is calcium-dependent, however, does not require for activation full ER calcium stores. Caspase-8 is activated independently of the intrinsic apoptotic pathway and does not require the increase of cytoplasmic calcium. In contrast, caspase-8 requires functional lysosomes for oxidative stress-induced activation in pancreatic acinar cells.


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Expression of ALS-related mutant SOD1 in astrocytes induces functional deficits in motoneuron mitochondria

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Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterised by the selective loss of motoneurons in the spinal cord, brainstem and motor cortex. Despite extensive research, the pathogenic mechanisms underlying motoneuron degeneration are far from understood. However, increasing evidence suggests that alterations in non-neuronal cell function are likely to contribute to the process of motoneuron degeneration (Gong et al. 2000; Pramatarova et al. 2001; Clement et al. 2003). In this study, the influence of glial cell genotype on motoneuron physiology was examined at a cellular level in an in vitro culture model based on the SOD1 mutation. Primary motoneurons from wild-type (WT) or transgenic mice carrying the SOD1G93A mutation were plated onto a layer of Primary motoneurons from wild-type (WT) or transgenic mice culture model based on the SOD1 mutation.

C86

Table 1

<table>
<thead>
<tr>
<th>% of motoneurons showing spontaneous cytosolic calcium activity</th>
<th>WT vs WTα</th>
<th>SOD1G93A</th>
<th>WT vs SOD1G93A</th>
<th>WT vs SOD1α</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Ca2+]cytosol (nmol SN fluorescence, arbitrary units)</td>
<td>0.39 ± 0.6</td>
<td>0.47 ± 0.6</td>
<td>3.5 ± 10.7</td>
<td>7.0 ± 2.1</td>
</tr>
<tr>
<td>∆ψm (TMRE fluorescence, arbitrary units)</td>
<td>2.14 ± 0.6</td>
<td>2.20 ± 0.6</td>
<td>2.5 ± 10.7</td>
<td>2.8 ± 2.1</td>
</tr>
<tr>
<td>Mean increase in NADH autofluorescence in response to mitochondrial inhibition arbitrary units</td>
<td>1092.7 ± 183.3</td>
<td>1067.4 ± 205.3</td>
<td>177.0 ± 71.1</td>
<td>171.0 ± 71.1</td>
</tr>
</tbody>
</table>

This work was supported by The Wellcome Trust and The Brain Research Trust.

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Disruption of myofibril organisation in the zebrafish mutant relaxed which lacks the dihydropyridine receptor β1a subunit

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Correct skeletal muscle function is essential for coordinated movement. The foundations of the skeletal muscle system are established in the embryo. Our previous studies have demonstrated a role for nerve activity in the regulation of myofibril organisation during zebrafish embryogenesis [1]. Homozygous zebrafish embryos of the relaxed (red257) mutant line are paralysed and die within days after hatching. The line carries a mutation in the β1a subunit of the voltage gated calcium channel, the dihydropyridine receptor, which is not expressed [2]. In the present study, we examined slow muscle fibre (adaxial cells) development in relaxed mutants, using whole mount immunocytochemistry. By 24 hours post fertilisation (hpf) adaxial cells had elongated and migrated to the lateral surface of the somite in both mutant and wild type embryos (n=4); a result indicative of calcium signalling via L-type calcium channel is not involved in these processes. In the wild type embryos the myofibrils are packed together into longitudinal bundles to form fibres, whilst in the mutant embryos myofibrils are not aligned laterally and appear disorganised. Previously we have shown that myofibril length was significantly increased relative to somite width in embryos from the nic1b107 line, which carries a mutation in the α-subunit of the nicotinic acetylcholine receptor. Here

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we report an apparent increase in the myofibril length of relaxed mutants (1.06±0.04 n=12 at 24 hpf and 1.08±0.06 n=12 at 48 hpf) compared to wild type embryos (1.03±0.04 n=12 at 24 hpf and 1.06±0.03 n=12 at 48 hpf). We conclude that calcium signalling via dihydropyridine receptors has a role in myofibril organisation during embryogenesis.


Funded by BBSRC.

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Hyposmotic cell swelling promotes cell division
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During cell cycle progression, cells increase in size and must pass a size-dependent checkpoint prior to mitosis. Once a cell has passed this restriction point, it is committed to one cycle of division. Swelling could act as a proliferative signal through activation of cytoplasmic signalling cascades and gene transcription [1]. Mitogen activated protein kinases (MAPK) are central components of the signalling pathways involved in cell cycle progression. Previous studies from this laboratory have demonstrated a rapid early increase in ERK-1,2 activity in chick myocytes following hyposmotically-induced swelling [2]. Such MAPK activation could provide a link between swelling and proliferation.

Embryonic chick hearts were removed at day 10 of development; myocytes were isolated enzymatically and maintained in culture. These cells may undergo at least one further cycle of cell division before terminal differentiation. Reducing extracellular osmolarity causes a significant increase in cell volume; e.g. cell volume increases to ~140% in hyposmotic solution (180 mosmol/l), before regulatory volume decrease occurs [2]. In this study, volume was perturbed by alteration of the extracellular osmolarity (150-400 mosmol/l by omission or addition of mannitol from the culture medium) for up to 48 hours. Cell proliferation was monitored by spectrophotometric assay of the number of viable cells [3]. Statistical comparison of absorbance between experimental groups was performed using one-way ANOVA and post-hoc Tukey tests; significant differences were accepted if p<0.05.

Our results demonstrate that cells exposed to hyposmotic media (200mosmol/l) showed a rapid early increase in proliferation, compared to control cultures (Fig. 1). The absorbance was significantly increased, relative to controls at 24 h (0.172±0.071; n=9 wells cf. 0.001±0.004; n=6) and 48 h (0.300±0.095; n=12 cf. 0.048±0.033; n=8). In contrast, cells incubated in hyperosmotic media (400mosmol/l; n=2 experiments) failed to proliferate. Similarly, cells treated with the MEK inhibitor PD98059 (50µM) also failed to proliferate in hyposmotic media (n=2 experiments). This study establishes a link between hyposmotically induced cell swelling and an increase in cell proliferation, and suggests a role for ERK1,2 signalling in swelling-induced mitosis. These data demonstrate that inducing changes in cell size may accelerate or halt progression through the cell cycle.
began. The secondary increase in ATP typically occurred 1 h after the start of Ca\(^{2+}\) oscillations and was inhibited by nocardazole which blocks meiotic resumption (n=12). The ATP levels slowly returned to prefertilization levels after the cessation of Ca\(^{2+}\) oscillations. The ATP increase at fertilization was dependent upon Ca\(^{2+}\) because fertilization of eggs that had been injected with the Ca\(^{2+}\) chelator BAPTA (n=9) blocked both Ca\(^{2+}\) oscillations and the rise in luminescence. Stopping ongoing Ca\(^{2+}\) oscillations at fertilization by chelation of extracellular Ca\(^{2+}\) caused a decline in ATP back to prefertilization levels (n=11). Furthermore, ATP increases could be stimulated by stimuli that cause Ca\(^{2+}\) oscillations such as addition of extracellular carbachol, or intracellular injection of PLC\(_{\text{zeta}}\) cRNA. Repetitive Ca\(^{2+}\) increases could also be induced by pulses of UV light delivered to eggs that had been injected with caged InsP\(_3\) (Jones & Nixon, 2000). Such repetitive InsP\(_3\)-induced Ca\(^{2+}\) increases stimulated at intervals of 10 min or 5 min resulted in a slow rise in luciferase luminescence (114 ± 7.9%, n=6, and 119 ± 8.7%, n=10, respectively). However, Ca\(^{2+}\) transients induced by InsP\(_3\) uncaging at intervals of 2.5 min resulted in a decrease in luciferase luminescence (-14.5 ± 5.9%, n=8). These data suggest that Ca\(^{2+}\) oscillations at fertilization cause a net rise in ATP that starts with the first Ca\(^{2+}\) transient.


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**PC178**

**The mitogen lysophosphatidic acid produces Ca\(^{2+}\) signals in mouse embryonic stem cells**

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Lysophosphatidic acid (LPA) is one of the major mitogens found in blood serum. This bioactive phospholipid mediates various cellular responses through G-protein coupled transmembrane receptors. In many cell types LPA is known to trigger an increase in intracellular Ca\(^{2+}\) concentration, which is involved in the regulation of cell proliferation. The control of the proliferative state in embryonic stem (ES) cells is of high significance in view of their potential clinical use. However, the signalling pathways involved in the maintenance of this state are still not fully understood. In the present study we investigated whether LPA, as an extracellular molecule, provoked an effect on Ca\(^{2+}\) mobilization in D3 mouse ES cells and studied the molecular mechanisms involved. Using the Fura-2 fluorescence technique we demonstrated that indeed LPA over the range of 100 nM to 10 µM activates Ca\(^{2+}\) signalling in 96% of the mouse ES cells examined (n=28 experiments). This Ca\(^{2+}\) signal was mediated by mobilization from internal stores given that Ca\(^{2+}\) release was reproduced in the absence of extracellular Ca\(^{2+}\) (n=10 experiments). Consisting with this finding, treating the cells with 1 µM thapsigargin, an inhibitor of the endoplasmic reticulum (ER) Ca\(^{2+}\) ATPase, completely abolished the LPA-stimulated Ca\(^{2+}\) increase in 3 out of 4 assays. Furthermore, we confirmed that in this LPA-induced Ca\(^{2+}\) mobilization the inositol 1,4,5-triphosphate (InsP\(_3\)) activation was involved as the specific phospholipase C (PLC) inhibitor U73122 (3 µM) completely attenuated the increase in calcium concentration (n=6). On the contrary, the inactive analogue U-73343 applied at the same concentration (3 µM) did not produce any effect (n=4). In addition, we saw that LPA effect could be exerted through at least two types of receptors, LPA-1 and LPA-2 whose mRNAs were detected by RT-PCR assays (n=4). On a next stage we shall focus on the effect of this calcium mobilization on ES cell proliferation, investigating the molecular mechanisms involved. The basic research in the area of stem cells will contribute to a better understanding of their proper characteristics and signal pathways, as well as proliferation and differentiation processes ensuring their better clinical application.

This work was supported in part by Grants from MCyT (GEN 2001-4748-C05-05; SAF2004-07483-C04-01) and Instituto Carlos III (GO3/171; GO3/210; GO3/212) to B.S. and from MEC (BFU2004-07283) to I.Q.

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**PC179**

**Effects of extracellular ATP on Ca\(^{2+}\) signalling in mouse pancreatic alpha-cells**

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The hyperglycaemic hormone glucagon, which is released from pancreatic islet alpha-cells, plays a critical role in the maintenance of blood glucose homeostasis. Several factors including nutrients, circulating hormones and neurotransmitters control glucagon secretion. The secretory process in these endocrine cells is triggered by a rise in intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)). In the present study, we have investigated the effects of the neurotransmitter and beta cell secretory product ATP on the Ca\(^{2+}\) signalling system of mouse alpha-cells within intact islets using confocal microscopy (1). Data were expressed as means ± SEM. Statistical significance was analysed using a Student’s t test (p<0.05). All cases were found significant.

Hypoglycaemic conditions (0.5 mM glucose), which stimulate glucagon secretion, induced oscillatory Ca\(^{2+}\) signals in alpha cells with a frequency of about 0.9 min\(^{-1}\). Addition of 100 µM of ATP markedly inhibited these low glucose-induced Ca\(^{2+}\) signals (62.3 ± 7.5% blockade, n=19). These results were reproduced in the presence of clonidine (70.7 ± 9.4%, n=11), a potent inhibitor of insulin release, suggesting that the effects on alpha-cells were not due to paracrine stimulation from neighbouring beta-cells. The inhibitory effects were also generated by both ADP and adeno-
We found that calmyrin interacts with the NH₂-terminus of InsP₃Rs in a Ca²⁺-independent manner (n=3). Video imaging of fura-2 loaded COS7 cells over-expressing YFP-tagged calmyrin revealed that calmyrin inhibited Ca²⁺ release from internal stores induced by purinergic agonist (ATP; 0.5, 1 and 100 μM) (refer to table 1 for percentage of responding cells, peak amplitude of the response and integrated response). A mutated protein in which the consensus site for myristoylation was altered (‘calmyrin-G2A’) also inhibited IICR. Unlike wild-type calmyrin, which was largely bound to cellular membranes, the calmyrin-G2A mutant was diffuse. From these data we concluded that calmyrin function is not dependent on its myristoylation and hence membrane targeting. Recombinant calmyrin also inhibited InsP₃-dependent Ca²⁺ flux from permeabilised cells (the IC₅₀ of IICR increased from 0.45 ± 0.06 μM to 0.87 ± 0.07 μM in the presence of 10 μM calmyrin (n=3)). The calmyrin-mediated inhibition of Ca²⁺ release was InsP₃R specific since there was no inhibition of caffeine-induced Ca²⁺ release from ryanodine receptors (peak amplitude of response to 0.5 mM caffeine: 59 ± 8.05 nM (n=10 calmyrin-expressing cells) vs. 67.5 ± 4.69 nM (n=42 control cells); p>0.01). To investigate the mechanism by which calmyrin mediates its effect we performed an InsP₃ binding assay using recombinant InsP₃R ligand binding domain. In these studies we demonstrated that calmyrin inhibited InsP₃ binding to the InsP₃R by 22 ± 3% (n=3), in a Ca²⁺-independent manner.

Our data indicate that calmyrin interacts with InsP₃Rs and inhibits IICR in a similar manner to CaBPs and CaM.

Table 1: Inhibition of IICR in COS7 cells stimulated with ATP

<table>
<thead>
<tr>
<th>AIP (μM)</th>
<th>Responders (%)</th>
<th>Peak amplitude (nM)</th>
<th>Integrated response (nM²·s⁻¹·μL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.7 ± 0.2</td>
<td>62.0 ± 10.7</td>
<td>87.6 ± 12.9</td>
</tr>
<tr>
<td>100</td>
<td>0.5 ± 0.05</td>
<td>11.0 ± 0.1</td>
<td>11.0 ± 0.1</td>
</tr>
<tr>
<td>10</td>
<td>0.5 ± 0.6</td>
<td>68.6 ± 13.5</td>
<td>68.6 ± 13.5</td>
</tr>
<tr>
<td>Calmyrin</td>
<td>0.5 ± 0.0</td>
<td>11.3 ± 1.5</td>
<td>11.3 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
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</tbody>
</table>


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**PC180**

**Inhibition of inositol 1,4,5-trisphosphate (InsP₃)-induced calcium release (IICR) by the calcium binding protein calmyrin**

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Inositol 1,4,5-trisphosphate receptors (InsP₃Rs) are widely-expressed intracellular channels that release Ca²⁺ from internal stores in response to many physiological stimuli. It is becoming apparent that InsP₃Rs form complexes with a multitude of accessory proteins. Many of the proteins that bind to InsP₃Rs appear to modulate channel function and Ca²⁺ release. InsP₃ Rs are known to be regulated by the ubiquitous EF-hand Ca²⁺-binding protein calmodulin (CaM). We recently demonstrated that members of the neuronal ‘calcium binding protein’ family (CaBPs), which also bind Ca²⁺ using EF-hand motifs and have approximately 50% homology with CaM, functionally interact with InsP₃ receptors (InsP₃Rs) and inhibit IICR (1). In the present study, we examined the putative interaction of another EF-hand-containing protein, calmyrin, which is ubiquitously expressed and has 56% homology to CaM.

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**PC181**

**The relationship between GSH level and compartmentation, telomerase activity and Bcl-2 expression in human mammary carcinoma cell lines Mcf7- and Mcf7+**

J. Markovic, C. Borras, A. Ortega, N. Mora, A. Broseta, F.V. Pallardo and J. Viña

*Physiology, University of Valencia, Valencia, Spain*

It has been proposed that cancer originates from a rare coincidence where the cell acquires simultaneously deregulated proliferation and suppressed apoptosis (1). The importance of GSH in the pathology of cancer has long been recognized, as well as
its central place in the control of vital cell processes of great diversity including cell proliferation (2). Bcl-2, the antiapoptotic protein, promotes the compartmentation of the GSH in the nucleus and thus contributes to the resistance to apoptosis (3). Considering the growing evidence that shows the importance of GSH compartmentation, and its role in numerous processes that occur in the nucleus, we have studied the changes in the GSH distribution throughout the cell cycle in MCF7- (Bcl-2 wild type +/-), and its Bcl-2 over expressing analogue, MCF7+ (Bcl2+/+) in order to see the possible relation between Bcl-2, GSH level, telomerase activity (TA) and cell proliferation. We studied the cell cycle by flow cytometry, total GSH (GSHt) level by spectrophotometry, telomerase activity by TRAP assay and its distribution by confocal microscopy. Triple staining was applied: propidium iodide (PI) to identify dead cells, Hoechst to localize nucleus and CellTracker green 5-chloromethylfluorescein diacetate (CMFDA), which marks GSH (specificity 95%). Our results show that the peak of GSHt in MCF7+ is at 6h (304.9 ± 8.5 nmol/mg prot, p<0.001 vs 12h) and precedes the peak of TA at 12h (2220 ± 754), while in MCF7- both GSHt (256.8 ± 61.1 nmol/mg prot, p<0.05 vs 24h) and TA (1448 ± 522) have their maximum at 18h after plating. MCF7+ cells show significantly higher level of GSHt and TA. By confocal microscopy we observed a growing tendency of GSH compartmentation in the nucleus of both cell types starting from 24h of culture which coincides with the plateau of high cell proliferation rate and is maintained until 72h after plating. We demonstrate that the level of GSH and TA in MCF7 cells could be, at least in part, Bcl-2 dependent. However, there was no significant difference in GSH distribution or in cell cycle dynamic and proliferation rate between MCF7+ and MCF7- cells. Green & Evan (2002). Cancer Cell 1, 19-30. Jones DP et al. (2002). Methods Enzymol 348, 93-112. Voehringer DW et al. (1998). Proc Natl Acad Sci USA Cell Biol 95, 2956-2960.

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**PC182**

**Induction of apoptosis by glutamine and serum deprivation in 3T3 fibroblast. Cell cycle and GSH compartmentation relationship**

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The glutamine (Gln) has been termed ‘conditionally essential’ because it is often depleted during times of catabolic stress like cancer, severe infection (sepsis) and burn injury. This characteristic depletion is attributed to its role as a metabolic intermediate aporting carbon and nitrogen for the synthesis of other amino acids, fatty acids, nucleic acids and proteins and also supplying a source of fuel for dividing cells. Gln is an essential component of glutathione (GSH) homeostasis. GSH is the primary intracellular antioxidant, scavenger of free radicals, peroxides and other reactive species. As a consequence of their exhaustion the process of apoptosis is triggered. Gln deprivation ultimately elicits apoptosis by intrinsic and/or extrinsic pathways, depending on the cell type (1). The aim of these work was to assess the glutamine and serum deprivation effects on the cell cycle and GSH compartmentation. Cells was cultured in DMEM media supplemented with 10% fetal calf serum (FCS) using 2x10^5 cells cm^2, and grown at 37°C in a humidified atmosphere of 5% CO₂ air mixture for 24 h. To induced apoptosis cultured medium was removed and the cells were cultured in Gln, serum and Gln- deprived medium for 48 h. In control cells medium was changed too. Cells were treated with propidium iodide (PI), to identify dead cells, and annexine V , for apoptosis measurement, then the of apoptosis and cells cycle were determined by flow cytometry. The GSH levels were determined by spectrophotometry. To visualize GSH localization, cells were observed with a Leica confocal microscope. Triple staining was applied: propidium iodide (Hoechst; 2 mg/ml) to localize nucleus and CellTracker green 5-chloromethylfluorescein diacetate (5mM; CMFDA), which marks GSH (specificity 95%). Our results show that the cell death and apoptosis levels are higher in the conditions of Gln deprivation, than in serum-deprived cells and control (33.6±3.41; 23±1.9 and 7.45±1.6, p<0.05, respectively). Also GSH levels in these cells were significantly lower compared with control cells and serum-deprived cells (7±28, 95±29.3 and 46±12.6 nmol/mg prot, p<0.05, respectively). By confocal microscopy we observed that serum deprivation caused nuclear GSH compartmentation, while in Gln-deprived cells the GSH distribution was homogeneous. Our results suggest that those cells with higher nuclear GSH levels are more resistant to apoptosis.

**Glutathione level and cell cycle relationship in embryonic cultures of Wistar rat neurons**

**J. Markovic, N. Mora, S. Ballester, P. Martin, A. Ortega, A. Broseta and F.V. Pallardo**

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Reduced glutathione (GSH) is a low molecular weight thiol and the most abundant non-enzymatic antioxidant in the cell. Its property to maintain cellular thiol/disulfide redox state gives it a central place in the control of great diversity of vital cell processes including cell proliferation (1). Our group has recently investigated the possible relationship between telomerase activity and cellular GSH concentration and we have demonstrated that the peak of total GSH (tGSH) in 3T3 fibroblasts coincides with the peak of telomerase activity (TA) at 24h in culture preceding the exponential phase of the cell growth (2). The objective of the present work was to analyse the relation between GSH levels and cell cycle in a non-proliferative cell model. We chose rat embryonic neurons that once differentiated do not have mitotic activity. Primary cultures were established of neurons from the cerebral cortex of fetal Wistar rats (gestation day 14) (3). To prevent non-
neuronal proliferation 20 µM cytosine arabinoside (AraC) was added on the 4th day of culture, and 24th afterwards half of the culture medium was changed. Until the 18th day of culture we studied the cell cycle by flow cytometry, GSH levels by spectrofotometry and cell viability by double staining propidium iodide to identify dead cells and Hoechst (2µg/ml) to localize all the nuclei. The level of glial contamination was defined by immunocitochemistry and was less than 3%.

In the first 4 days of culture, before the AraC was added, the peak of GSH at 48h (39.6±4.6 nmol/mg prot, p<0.01 and p<0.05 vs 3h and 7 days, respectively) was followed by the maximum proliferation at 72h (S+M/G2=15±1.1, p<0.01 vs. 5.7±0.3 at 7 days). After the AraC was added, the cell culture stabilized in G0/G1 (39.2± 0.7%) and the level of GSH dropped significantly (27.9±2.5 nmol/mg prot at 7 days). A higher percentage of cell death could be attributed to the elimination of non-neuronal cells by AraC.

Our results show that while freely proliferating, the primary culture of cerebral cortex demonstrates the same tendency as in 3T3 fibroblasts (2). The GSH maximum level precedes the peak of proliferation. However, in a 97% pure neuronal culture, when the non-neuronal cells were eliminated, the level of GSH and proliferation is significantly lower and it remains stable throughout the period of culture.


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PC184

Wheat germ agglutinin holds nuclear pores of chick embryo sensory neurones open for calcium diffusion

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Although calcium is thought to equilibrate across the nuclear envelope, the envelope nevertheless represents a significant barrier to rapid diffusion. We have investigated the delay to calcium diffusion caused by the nuclear envelope, and the effect of two agents on that delay: histone proteins, which will be actively transported by the pore complex, and wheat germ agglutinin, which inhibits active pore transport.

Cultured sensory neurones of 12 day old chick embryos replated onto a polyornithine substrate on the day before experimentation displayed a simple ovoid shape without neurites. Immediately before experimentation nuclei were stained with 2 µM Hoechst 33342. The bathing medium was then switched to 120 mM NaCl, 1.2 mM MgCl2, 5.5 mM KCl, 1.8 mM CaCl2, 1.8 mM TEACl, 10 mM HEPES, 25 mM glucose, 1 µM tetrodotoxin and 5 µM (S)-(−)-BAY K 8644. The presence of BAY K enhanced calcium currents and therefore increased the depolarization-evoked fluorescence signal. Neurons were whole cell patch clamped, at room temperature, on the stage of a Zeiss 510 confocal microscope through pipettes containing 125 mM CaCl2, 4 mM MgATP, 10 mM HEPES and 100 µM of the calcium indicator Oregon Green BAPTA 488 Dextran, 10 kD, pH 7.2, plus 500 µg/ml of a histone mixture (Sigma H-7755) or 100 µg/ml of wheat germ agglutinin (Sigma L-9640) as appropriate.

Neurones were held in whole cell mode for 3 min to allow diffusion of the dye and active agents into the cytosol and to the nuclear pores. Neurones were then scanned every 1.92ms along a line that passed through the nucleus (excitation 488 nm, emission >505 nm, confocal plane 3 µm). Depolarization (-70 mV to +10 mV for 50 ms) evoked a calcium increase that could be seen to pass from the cell edge to the cell centre within tens of milliseconds. Comparing the rising phase of the fluorescence signal in the nucleus, and at the same distance from the cell edge in the cytosol, yielded a value for the delay due to the nuclear envelope. In control cells this was 6.2 ± 2.7 ms (± SEM, N=10).

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PC185

SNAP-25 is a novel inositol 1,4,5-trisphosphate receptor (InsP3R) interacting protein that regulates intracellular Ca2+ release and influx

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Inositol 1,4,5-trisphosphate receptors (InsP3Rs) are major intracellular Ca2+ release channels in non-excitable cells. In addition to regulation by InsP3 and Ca2+, they are modulated by post-translational modification and the binding of accessory proteins. Thus, InsP3Rs act as scaffolds, anchoring many signalling proteins to the site of Ca2+ release, where they either regulate, or are regulated by Ca2+ release through the channel (1). To identify novel InsP3R interacting proteins, a proteomic approach was used. To this end, InsP3Rs were immunoprecipitated from rat brain lysate, which is a rich source of type 1 InsP3R. Co-immunoprecipitated proteins were subsequently eluted and subjected to 2-dimensional liquid chromatography and Matrix Assisted Laser Desorption Ionization Time-of-flight Mass Spectrometry analysis (MALDI-TOF). Using this strategy, we identified the SNAP-25-associated protein SNAP-25. This interaction was confirmed by subsequent immunoblotting of proteins co-immunoprecipitated with the InsP3R (n>3). SNAP-25 is a protein involved in Ca2+-regulated secretory processes and associates with N- and P/Q-type voltage-operated plasma membrane Ca2+-channels.
To test whether the expression of SNAP-25 affects Ca^{2+} signalling, we established stable HeLa cell lines that expressed YFP-SNAP-25 in a tetracycline-inducible manner. In these cells, YFP-SNAP-25 was targeted to the plasma membrane. Using fura-2 imaging, we investigated whether overexpression of YFP-SNAP-25 affected InsP_3-induced Ca^{2+} release and store operated Ca^{2+} entry. Cells overexpressing YFP were used as control. Experiments were performed on at least two different days and on three coverslides for each cell line per day. Statistical significance was determined by a Mann-Whitney U test. Data are presented as mean±SEM.

To investigate the effect of SNAP-25 on InsP_3-induced calcium release, cells were stimulated with increasing doses of the InsP_3-generating agonist histamine (0.5, 1 and 100 μM). Cells overexpressing YFP-SNAP-25 exhibited decreased Ca^{2+} responses to all concentrations of histamine applied (78.6±5.7%, 74.5±6.5%, and 73.3±1.7%, p<0.01 for all three concentrations). The rate of quench of cellular fura-2 fluorescence by Mn^{2+}, which affects both Ca^{2+-}release from the ER and SOCE. In summary, we have shown that SNAP-25 is a novel InsP_3R interacting protein, which affects both Ca^{2+-}release from the ER and SOCE.


Where applicable, the authors confirm that the experiments described here conform with the Physiological Society ethical requirements.

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PC186

Genetic iron overload versus dietary iron overload in SWR mice

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Hereditary haemochromatosis is an autosomal recessive disorder of iron (Fe) metabolism and a mutation in the Hfe gene is the most common cause of the disease. Haemochromatosis is characterised by defective regulation of dietary iron absorption that leads to excessive Fe accumulation in various organs including the liver, pancreas, heart, joints, and pituitary gland leading to hepatic cancer, liver failure, diabetes, and heart disease. Recent findings suggest hepcidin, a hormone produced by the liver, is a negative regulator of Fe absorption and is itself regulated by iron, anaemia, hypoxia and inflammation. In the case of haemochromatosis expression of hepcidin is inappropriate for the levels of liver Fe and this leads to increased Fe uptake despite liver iron being elevated. These observations are based on measurement of iron and hepcidin expression in biopsies or a part of a lobe; however, there is no information on Fe levels or hepcidin expression in relation to different lobes in the liver.

The aim of this study was to investigate the level of Fe in each lobe of the liver and measure the hepcidin gene expression in SWR-wild type (wt) and SWR-Hfe knockout mice.

Both wt and Hfe knockout mice were studied at the age of 12 weeks. The livers were divided into five lobes and named A to E, Fe quantification was done by the Torrance and Bothwell method and hepcidin gene expression was measured by RT-PCR.

We found that in SWR wt mice liver Fe in all the lobes ranged from 70 to 90 μg/g dry weight (79.8±5.1 μg/g dry weight). In SWR Hfe knockout mice, liver Fe levels were higher as expected and in lobes B to E Fe levels ranged from 350 to 500 μg/g dry weight (438±15.6 μg/g dry weight). Interestingly, lobe A had a much higher level of Fe than any of the other lobes (821.6±102.8 μg/g dry weight, n=8). When hepcidin 1 gene expression levels were measured, there was no difference between the different lobes in the SWR wt mice (Table 1), but in the SWR Hfe knockout mice, although the hepcidin levels were lower than the wt, there were differences between the lobes, lobe A being the one with the highest level of hepcidin expression.

When SWR wt mice were fed a high Fe diet, although there was a great accumulation of Fe in the liver, no differences were seen between the lobes (Table 2).

In conclusion, the Hfe gene seems to cause a preferential accumulation of Fe in the specific lobes of the liver. Furthermore hepcidin expression was highest in the lobe with the highest Fe levels. This suggests that liver Fe levels regulate hepcidin but this regulation is perturbed by Hfe. This finding is also relevant to the diagnosis of haemochromatosis as the common practice is to perform biopsies of a particular region of the liver and this might not correspond to the actual Fe accumulation status of the liver.

Table 1. Hepcidin 1 mRNA content relative to actin, in the different liver lobes of SWR wt (n=5) and Hfe knockout (n=5) fed on a normal diet

<table>
<thead>
<tr>
<th>Lobe</th>
<th>SWR wt (hepcidin 1 expression)</th>
<th>SWR Hfe KO (hepcidin 1 expression)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.25±0.06</td>
<td>0.14±0.10</td>
</tr>
<tr>
<td>B</td>
<td>0.21±0.03</td>
<td>0.04±0.03</td>
</tr>
<tr>
<td>C</td>
<td>0.31±0.07</td>
<td>0.04±0.00</td>
</tr>
<tr>
<td>D</td>
<td>0.12±0.02</td>
<td>0.06±0.03</td>
</tr>
<tr>
<td>E</td>
<td>0.12±0.02</td>
<td>0.02±0.00</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

Table 2. Liver irons (μg/g dry weight) of the different lobes in SWR wt fed an high iron diet (n=6)

<table>
<thead>
<tr>
<th>Lobe</th>
<th>SWR wt on high iron diet (μg/g dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>57.8±10.7</td>
</tr>
<tr>
<td>B</td>
<td>484.7±55.4</td>
</tr>
<tr>
<td>C</td>
<td>512.7±48.4</td>
</tr>
<tr>
<td>D</td>
<td>514.3±104.4</td>
</tr>
<tr>
<td>E</td>
<td>527.4±79.1</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

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Adhesion of human breast cancer cell lines: role of voltage-gated sodium channel β1 subunit

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Voltage-gated Na+ channel (VGSC), predominantly neonatal Nav1.5 (nNav1.5), expression is upregulated in metastatic breast cancer (BCa) and VGSC activity potentiates a variety of metastatic cell behaviours (Fraser et al. 2005). VGSCs incorporate one or more β-subunit (VGSCβ) which can modulate channel gating and functional expression (Chen et al. 2002). Interestingly, VGSCβ can also serve as cell adhesion molecules by interacting with extracellular matrix proteins and cytoskeleton (Isom, 2002). The aims of the study were (1) to determine which VGSCβ were expressed in weakly and strongly metastatic human BCa (MCF-7 and MDA-MB-231, respectively) cell lines and (2) to test the possible role of VGSCβ expression in cellular adhesion and migration.

MCF-7 and MDA-MB-231 cells were cultured (Fraser et al. 2005). Real-time PCR and Western blot (WB) with a polyclonal antibody specific for β1 (Malhotra et al. 2000) were used to study and quantify gene and protein expression. Transwell migration and adhesion were measured as before (Fraser et al. 2005; Aydar et al. 2006). A small interfering RNA (siRNA) technique was used to silence specifically the β1 gene in MCF-7 cells; the control transfection involved a scrambled siRNA (siControl). Data (mean ± SEM) were analysed by paired t tests, unless otherwise stated.

Real-time PCR revealed that in both MCF-7 and MDA-MB-231 cell lines, VGSCβ expression profile was as follows: β1>> β4> β2 (β3 was absent). MCF-7 cells had a much higher overall level of VGSCβ mRNA expression, compared to MDA-MB-231. WBs verified that β1 protein was strongly expressed in MCF-7 and barely detectable in MDA-MB-231. In siRNA-transfected MCF-7 cells, β1 mRNA (normalised to cytome b5 reductase) was reduced by 76 ± 4% after 4 days, relative to siControl (P<0.01; n=3). Protein-level reduction (normalised to actin) was less pronounced, only 39 ± 7% after 8 days (P<0.01; n=1). Under control conditions, MCF-7 cells were much more adhesive than MDA-MB-231 cells, in line with their weak metastatic potential. However, 8 days after β1 siRNA transfection, the adhesion of MCF-7 cells was reduced by 34 ± 2% (P<0.001; n=6). Interestingly, concurrently, the cells' migratory activity increased by 121 ± 14% compared to siControls (P < 0.05; n = 8). This increase was largely dependent upon VGSC activity since application of TTX (10 microM) to siRNA-treated cells reduced migration to the control level (P = 0.4 for siRNA vs siControl, both treated with TTX; n=8). Indeed, in siRNA-treated MCF-7 cells, nNav1.5 mRNA and protein levels were significantly higher.

We conclude (1) that VGSCβ1 subunit expression makes a significant contribution to the strong adhesiveness of the weakly metastatic MCF-7 cells and (2) that VGSCβ1 downregulation would increase migration via VGSC expression/activity.


Role of phospholipases in store-operated Ca2+ entry in human platelets

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Store-operated Ca2+ entry (SOCE) is a major pathway for Ca2+ entry in human platelets, although the mechanism of SOCE activation has not been clearly established. In one model, Ca2+ store depletion induces de novo conformational coupling of the type II IP3 receptor to hTRPC1, believed to be a component of the store-operated channel. This coupling requires basal levels of IP3 and functional IP3 receptors (1, 2). In contrast, an alternative model suggests that Ca2+ store depletion induces formation of a Ca2+ influx factor (CIF) that displaces calmodulin (CaM) from Ca2+-independent phospholipase A2 (iPLA2), resulting in iPLA2 activation and lysophospholipid production, which then activates the store-operated channel (3). Here the role of iPLA2 in SOCE activation in human platelets has been investigated.

Fura-2–loaded human platelets were stimulated with the CaM inhibitor, calmidazolium (CMZ), resulting in a concentration-dependent increase in Ca2+ release and Ca2+ entry. At no concentration was Ca2+ entry seen without Ca2+ release. No Ca2+ entry was detected using 1 μM CMZ, and only a relatively small Ca2+ entry was detected using 10 μM. In contrast, iPLA2 was strongly activated by 1 μM CMZ, and maximally activated by 10 μM. These data suggest that iPLA2 activation is not sufficient for SOCE activation in these cells.

Thapsigargin–evoked SOCE was completely abolished by prior incubation with the iPLA2 inhibitor, bromoenol lactone (BEL; 25 μM; 30 min). BEL was found to increase resting intracellular Ca2+ concentration from 61 ± 4 nM to 155 ± 2 nM (mean ± SEM; n = 6; p < 0.005; Student’s t test). BEL also reduced PAR-2–dependent Ca2+ release to 20.2 ± 2.1% of control (n = 6; p < 0.005), which appeared to be due to inhibition of agonist-evoked IP3 synthesis. IP3 levels in cells stimulated by the PAR-1 agonist, SFLLRN, after BEL treatment were not significantly higher than basal (100.9 ± 10.2% of control; n = 6; p = 0.48), as measured by a fluorescence polarisation assay.

Inhibition of phospholipase C by Et-18-OCH3 also significantly inhibited TG-evoked SOCE to 12.7 ± 3.6% of control (n = 9; p < 0.001). This is consistent with an essential role for basal IP3 in the activation of SOCE (1). The disruption of IP3 production

Isom LL (2002). Front Biosci 7, 12-23.


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by BEL may be owing to the role of iPLA$_2$ in remodelling of the fatty-acid content of membrane phospholipids. In conclusion, although iPLA$_2$ appears to have an important role in regulation of cell signalling through the remodelling of cellular phospholipids, it is unlikely to play a direct role in SOCE activation in human platelets.


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**PC191**

**Transforming growth factor-$\beta$1 induces heme oxygenase-1 in human aortic smooth muscle cells: role of Nrf2, mitogen-activated protein kinases and superoxide generation**

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Reactive oxygen species contribute to atherogenesis resulting from endothelial and smooth muscle dysfunction. We have previously reported that oxLDL induces the expression of the antioxidant-like stress protein heme oxygenase-1 (HO-1) in human aortic smooth muscle cells (HASMC, Anwar et al. 2005). HO-1 catalyses the pro-oxidant heme to the antioxidants biliverdin and the vasodilator carbon monoxide (Siow et al. 1999). Transforming growth factor-$\beta$1 (TGF-$\beta$1) stimulates vascular SMC growth and extracellular matrix synthesis which contributes to vascular remodelling (Topper, 2000). Induction of cytoprotective antioxidant genes such as HO-1 is regulated by the transcription factor Nrf2 and act to limit oxidative injury (Ishii et al. 2000). We have now investigated whether TGF-$\beta$1 activates Nrf2 to mediate HO-1 induction and the involvement of mitogen-activated protein kinases (MAPK) and free radical generation in human aortic SMC. Cells were treated with TGF-$\beta$1 (0–10 ng/ml, 0–24 h) and HO-1 expression and phosphorylation of extracellular signal-regulated kinase (ERK1/2), p38MAPK and c-Jun N-terminal kinase (JNK) determined by western blot analyses. Activation of Nrf2 translocation was determined by immunofluorescence and immunoblot analyses of nuclear lysates. NADPH-dependent superoxide generation was assessed by lucigen chemiluminescence in cell homogenates. The statistical significance of quantified data were evaluated using Student’s unpaired t tests.

TGF-$\beta$1 (2.5 ng/ml, 2 h) treatment led to nuclear translocation of Nrf2, phosphorylation of JNK, p38MAPK and ERK1/2, and increased superoxide production by 1.5±0.2-fold (mean±SEM, n = 3, p<0.01). Pretreatment of cells with apocynin (100 µM), an NADPH oxidase inhibitor, significantly attenuated superoxide generation elicited by TGF-$\beta$1 by 34±8% (p<0.05, n=3). HO-1 expression was enhanced by TGF-$\beta$1 (2.5 ng/ml), reaching a maximum 10.3±2.3-fold increase between 8–12 h (mean±SEM, n = 3, p<0.01).

Modulation of TGF-$\beta$1-mediated Nrf2 activation, free radical production and HO-1 expression may provide mechanistic insights for the contribution of this growth factor to vascular disease processes.


Supported by the Guy’s & St Thomas’ Charitable Foundation and the British Heart Foundation.

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**PC191a**

**Activity of protein tyrosine phosphorylation enzymes in stomach mucosa cells of rats with stress-induced gastric lesions**

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(Title only)

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