Ionomycin causes opacities in cultured sheep lenses by damaging the outer layers of the cortex

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Background

Cortical cataracts have been associated with increased levels of calcium[1]. A model has been developed for cataracts by increasing the calcium levels in cultured sheep lenses using the calcium ionophore, ionomycin[2]. This model has been used to test the efficacy of potential therapeutic agents for cataracts, particularly calpain inhibitors[3]. The calcium-dependent calpains have been implicated in cataract formation[4], and are known to cleave lens crystallins and cytoskeletal proteins.

Purpose

The purpose of these experiments was to determine which parts of the lens were affected by ionomycin and to look for specific evidence of calpain activation during opacification.

Methods

Lamb lenses were dissected from whole eye globes and cultured in Eagles minimum essential medium (EMEM) for 48 hr. Control lenses (n=6) remained in EMEM for a further 4 days. Lens opacity was induced in the test group (n=6) by exposure to 2μM ionomycin. The requirement for calcium in the medium was tested using lenses treated with ionomycin and 5mM EGTA (n=6). Remaining lenses were dissected into epithelial, cortex and nuclear fractions. The calcium concentration in each fraction was determined by flame emission spectroscopy and the calpain activity by casein zymography. Proteolysis of lens proteins was assessed by two-dimensional electrophoresis (2-DE) and Western blotting of the cytoskeletal proteins spectrin and vimentin. Spots from 2-DE gels were identified by mass spectrometry and by comparison with previously mapped lens proteins.

Results

Progress of Opacification

Control lenses cultured in EMEM remained transparent over the 4-day period (Figure 1). Ionomycin treatment initially caused opacification at the lens equator which became more generalized with time. Dissection of these lenses showed that a liquid fraction had developed between the epithelium and the cortex. Chelation of calcium with EGTA reduced but did not prevent opacification. Ionomycin treatment increased in lens calcium concentration in the epithelial, cortex and liquid fractions to levels similar to that in the medium (1.8mM).

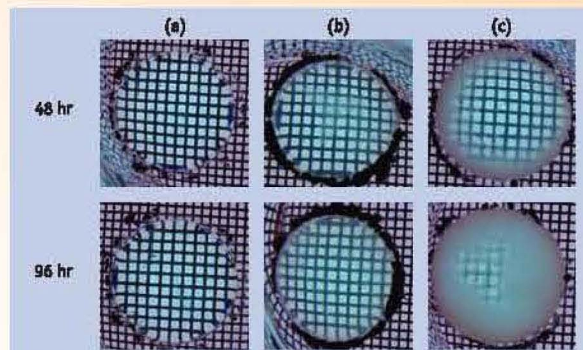


Figure 1 Bright field images of typical cultured lenses from (a) EMEM only, (b) Ionomycin + EGTA, (c) ionomycin only, taken after 48 and 96 hours of treatment.

Calpain 2 Activation

Calpain activity was detected in the soluble epithelium and liquid fractions at 96 hr of ionomycin treatment by casein zymography (Figure 2). The upper band in each lane represents intact calpain 2. The lower band indicates autolysed calpain after its previous activation. These results show that calpain 2 activation in the outer regions of the lens was associated with ionomycin treatment while the EGTA prevented activation. Analysis of the liquid fraction revealed mainly autolyzed calpain 2.

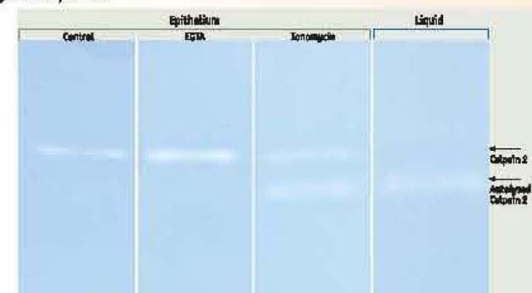


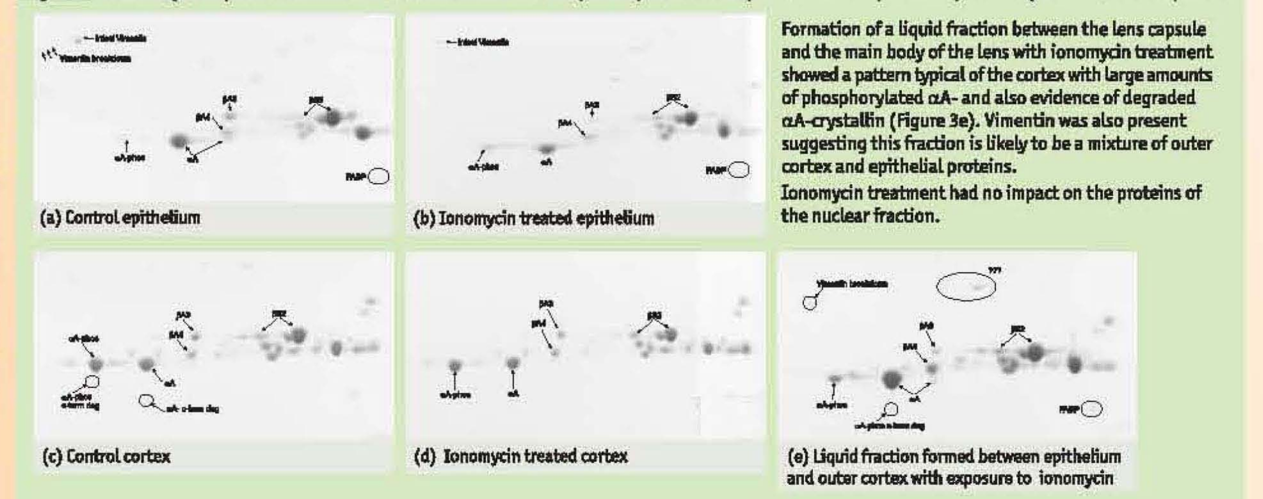
Figure 2 Calpain activity in the epithelium and liquid fractions of cultured ovine lenses. Each sample lane has 25μg total soluble protein.

Changes in Lens Proteins

Each soluble lens fraction was analysed by 2-DE. Ionomycin treatment caused large changes in epithelial crystallins with increased phosphorylation of αA-crystallin and a decrease in both the major αA- spot and a smaller more basic spot. ßA3- and ßA4- crystallin were also reduced (Figure 3a and b). There was also evidence of the loss of vimentin and the fatty acid binding protein.

No notable changes were detected in the protein profile of the cortex region between normal and ionomycin treated lenses (Figures 3c and d). However, a c-terminally degraded form of αA-crystallin and phosphorylated αA-crystallin were present in this fraction. A much greater proportion of the αA-crystallin in the cortex was phosphorylated compared to the epithelium.

Figure 3 2-DE analysis of protein fractions from lenses cultured in EMEM (control) for 96 hr compared to those exposed to 2μM ionomycin over the 96 hr period.



(a) Control epithelium

(b) Ionomycin treated epithelium

(c) Control cortex

(d) Ionomycin treated cortex

(e) Liquid fraction formed between epithelium and outer cortex with exposure to ionomycin

Formation of a liquid fraction between the lens capsule and the main body of the lens with ionomycin treatment showed a pattern typical of the cortex with large amounts of phosphorylated αA- and also evidence of degraded αA-crystallin (Figure 3e). Vimentin was also present suggesting this fraction is likely to be a mixture of outer cortex and epithelial proteins.

Ionomycin treatment had no impact on the proteins of the nuclear fraction.

Changes in Vimentin

Detection of vimentin in the soluble lens epithelium and cortex was used to confirm its protolysis on the 2-DE gels (Figure 4). There was evidence of vimentin proteolysis in the epithelium from control lenses. This was prevented by EGTA treatment which is consistent with a role for calpain in this proteolysis. Ionomycin treatment lead to a decline in the amount of vimentin and its fragments.

The cortex fraction consisted largely of intact vimentin in all treatments. However there was much less vimentin in the lenses treated with ionomycin. The liquid fraction between the cortex and epithelium had only fragments of vimentin while vimentin was undetectable in the nucleus.

Changes in Spectrin

Spectrin and its breakdown products were determined by Western Blotting of the soluble lens fractions (Figure 5). The epithelial tissue showed extensive proteolysis of native spectrin (280 kDa) to 150 kDa fragments in all treatments. This proteolysis may be attributed to either calpain or caspase 3. The control and ionomycin treatments also revealed the 145 kDa fragment characteristic of calpain proteolysis but this fragment was not present following treatment with EGTA. In the cortex the 150 kDa spectrin breakdown product appeared to increase with ionomycin treatment. The liquid fraction did not possess intact spectrin but had both the 150 and 145 kDa breakdown products. Disappearance of intact spectrin in the nucleus was not retarded by EGTA treatment suggesting this part of the lens was not affected solely by ionomycin treatment. This observation is also supported by the absence of change in the 2-DE pattern for the nucleus.

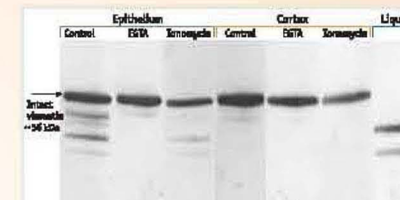


Figure 4 Western detection of vimentin (V9 clone, Santa Cruz) in soluble protein fractions from lenses cultured in EMEM, EGTA and ionomycin for 96 hr.

Figure 5 Western detection of spectrin (α-fodrin, Affiniti) in soluble protein fractions from lenses cultured in EMEM, EGTA and ionomycin for 96 hr.

Conclusions

Ionomycin caused opacification of cultured sheep lenses and opacification was associated with a rise in lens calcium. The epithelium and the outer cortex were most affected by ionomycin treatment with no evidence of change in the lens nucleus. The formation of a liquid fraction between the lens epithelium and outer cortex following ionomycin treatment indicated protein degradation in this region. Spectrin and vimentin proteolysis and changes in crystallins were prominent features of this opacification model. These changes were consistent with calpain activation. However the limited changes in the inner cortex and the absence of changes in the nuclear fraction implied that ionomycin had not penetrated to the interior of the lens.

References

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