Imbalance between pro-apoptotic and pro-survival factors in human retinal pericytes in diabetic-like conditions

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Running head: Pericyte apoptosis in diabetic-like conditions

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Abstract

Purpose: Loss of pericytes is one the key-events in the pathogenesis of diabetic retinopathy. We have previously demonstrated that human retinal pericytes (HRP) are more vulnerable to intermittent than stable high glucose concentrations, with an increase in apoptosis. Our aim was to explore the expression of molecules involved in pro-apoptotic and survival pathways in pericytes cultured in stable/intermittent high glucose and/or hypoxia, to clarify the mechanisms of action of these diabetic-like stressing stimuli.

Methods: HRP were exposed intermittently at 48-hr intervals to high (28.0 mM)/physiological (5.6 mM) glucose for 8 days (intHG) and/or hypoxia over the last 48 hrs. Control cells were kept in stable physiological and high glucose. Cell proliferation and apoptosis were assessed. The expression of pro-apoptotic and pro-survival molecules was evaluated by Western blotting. Caspase-8 translocation from the cytoplasm into the nucleus was checked by immunofluorescence.

Results: Hypoxia, alone and combined with intHG, increased HRP apoptosis and decreased proliferation. Pro-apoptotic molecules were significantly increased in HRP cultured in these conditions, while some survival markers decreased. Conversely, in stable HG, pro-apoptotic molecules were stable or even decreased, and survival factors increased. Translocation of caspase-8 from cytoplasm into nucleus indicates a primary role for this molecule in inducing apoptosis.

Conclusion: Diabetic-like conditions are able to stimulate pericyte apoptosis through activation of pro-apoptotic molecules, leading to an imbalance between pro-apoptotic and survival signalling pathways, with caspase-8 playing a pivotal role. Our identification of such intermediates could help finding new therapeutic approaches for the prevention of diabetic retinopathy.

Keywords: diabetic retinopathy, pericyte(s), apoptosis, apoptotic pathways, caspase-8

Introduction

Pericytes modulate vascular permeability, including the blood-brain and blood-retinal barriers, and regulate endothelial cell (EC) proliferation, migration and survival (Armulik et al. 2005). Early loss of retinal capillary pericytes, together with thickening of the basement membrane, is one key-event in the pathogenesis of diabetic retinopathy (DR), as it may lead to failure of control on endothelial proliferation and, consequently, to abnormal angiogenesis (Gerhardt & Betsholtz 2003; Armulik et al. 2005).

Although intervention studies have clearly linked severity of the microvascular complications of diabetes with duration and severity of hyperglycaemia (DCCT 1993; UKPDS 1998), other factors may play a role in their pathogenesis. In particular, hypoxia caused by capillary closure and non-perfusion is a recurrent condition in the diabetic retina, leading to increased production of vascular endothelial growth factor (VEGF), the primary cause of proliferative DR (Nyengaard et al. 2004). It is well understood that loss of pericytes is primary due to the effects of hyperglycaemia (Beltramo & Porta 2013), but, more recently, a contribution of hypoxia in the pericyte loss has also been addressed (Aplin et al. 2016). We have previously demonstrated that human retinal pericytes (HRP) are more vulnerable to intermittent than stable high glucose concentrations, with an increase in apoptosis (Beltramo et al. 2009a; 2009b). Activation of death receptors and mitochondrial injury caused by stress stimuli have been associated with cell damage and apoptosis in the retina of diabetic subjects with DR (Valverde et al. 2013). Nevertheless, the activation of these signalling pathways in pericytes is poorly understood.

Our purpose was therefore to investigate the effects of hypoxia combined with hyperglycaemia on HRP and to identify the pro-apoptotic and survival markers involved in pericyte damage. Identification of such intermediates could help to unravel new therapeutic approaches for the prevention of DR.

Materials and methods

Cell cultures

Home-stabilized HRP (Berrone et al. 2009) were used as a model. HRP were cultured in DMEM supplemented with 10% fetal bovine serum (Sigma Aldrich, St Louis, MO, USA), at 5.6 mmol/L D-glucose concentration (physiological condition, NG). High glucose concentrations (HG) were obtained by adding D-glucose to a final concentration of 28 mmol/L. Cells were also grown in intermittent HG conditions (48hr HG/ 48hr NG twice, intHG). All conditions were maintained for 8 days. Hypoxic conditions (hypo) were obtained by keeping cultures in a 5%CO_2 / 94%N_2 /1%O_2 gas mixture for the last 48 hrs.

Cell survival parameters

HRP were counted in Bürker chambers by 2 independent operators after Trypan blue staining. Proliferation was measured as BrdU incorporation (*Cell Proliferation ELISA BrdU kit,* Roche Diagnostics, Basel, Switzerland) and apoptosis as DNA fragmentation (*Cell Death Detection ELISA^{PLUS} kit*, Roche). Results were checked by a fluorescent/chemioluminescent assay which measures viability, cytotoxicity and apoptosis in the same well (*ApoTox-Glo™ Triplex Assay*, Promega Corporation, Madison, WI, USA). All procedures were carried out according to manufacturers' instructions.

Western blot analysis

Cells were lysed using M-PER Mammalian Protein extraction reagent (ThermoFisher Scientific, Waltham, MA, USA) added with 10 µl/ml protease inhibitor cocktail kit (ThermoFisher). Extracts were kept ice-cold and cleared by centrifugation at 20,000 *g* for 15 min at 4°C. The supernatant was aliquoted and stored at −80°C. Protein concentration was measured using Bradford method. 30 µg of total protein were loaded on sodium dodecyl sulfate polyacrylamide gel and separated by electrophoresis. Gels were transferred to Immobilon membranes (Merck-Millipore, Billerica, MA, USA). Membranes were blocked using 5% non-fat dried milk in 10 mmol/l Tris-HCl and 150 mmol/l NaCl pH 7.5 and incubated overnight with relevant antibodies in 0.05% Tween-20, 10 mmol/l Tris-HCl, and 150 mmol/l NaCl pH 7.5. All primary antibodies were used at a 1:1000 dilution. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) Western blotting protocol (Millipore). Antibodies used: anti-FasL (AB-16982) from Merck-Millipore; anti-Bim (Ref. 559685) and anti-BclxL (Ref. 551269) from BD Biosciences PharMingen (San Diego, CA); anti-Bid (Ref. AF860) from R&D Systems (Minneapolis, MN, USA); anti-cleaved (Asp175) caspase-3 antibody (Ref. 9661), anti-phospho MAPK (Ref. 9101), anti-Puma (Ref. 14570) and anti-caspase-9 (ref 9504) from Cell Signaling (Beverly, MA, USA); anti–phospho-Akt1/2/3 (Ser473; Ref. sc-7985-R), anti-Akt1/2/3 (Ref. sc-8312), anti-caspase-8 (Ref. sc-7890), anti-PCNA (Ref. sc-56) and anti-Bax (Ref. sc-493) from Santa Cruz Biotechnology (Palo Alto, CA, USA); anti-calpain-2 (C3989) and anti-α-tubulin (T-0198) from Sigma-Aldrich.

The relative signal strength was quantified by densitometric analysis (ImageJ software, NIH, USA), and values normalized against α-tubulin. As regards activated proteins, results were expressed as the ratio of active to pro/inactive forms: active caspase-8/pro-caspase-8 and truncated Bid (tBid)/Bid.

Immunofluorescence staining

Immunofluorescence was performed after fixation of cells with ice-cold methanol, by overnight incubation at 4°C with an anti-cleaved-caspase-8 (Asp391) antibody (1 µg/ml) (Ref. 9496, Cell Signaling). Secondary antibody was a FITC-conjugated goat anti-rabbit IgG (Sigma Aldrich) used at a 1/1000 dilution for 1h. DAPI was used to blue-stain the cell nuclei. Images were taken under a Leica DM 2000 microscope (Leica, Wetzlar, Germany), equipped with a Leica DFC 320 camera and Leica QWin Plus 2003 digital processing and analysis software.

Statistical analysis

Results are expressed as mean ± SD of 5 independent experiments, normalized against control (NG). Statistical comparisons among groups were carried out by two-tailed Student's t-test for paired data or Wilcoxon's Signed Ranks test, as appropriate. Results were considered significant for $p \leq 0.05$.

Results

Our results show that hypoxia is able to decrease HRP proliferation (-24%, p=0.002 vs NG) and increase their apoptosis (+19%, p=0.045), similarly to intHG, and has a synergic effect with the latter (proliferation: -24%, p=0.001, and apoptosis: +42%, p=0.003 vs NG) **(Fig. 1a-b).**

We investigated the apoptotic and survival molecules that were activated and/or decreased under these stress experimental conditions. Regarding pro-apoptotic markers, we investigated the expression of: Fas ligand (FasL), Bid and its truncated active form (tBid), Bim, Bax, p53, p53 upregulated modulator of apoptosis (PUMA), calpain-2, pro/active caspase-8, and caspase-9, while the pro-survival factors examined were: phosphorylated Akt (pAkt Ser473), phosphorylated mitogen-activated protein kinase (pMAPK), BclxL, and proliferating cell nuclear antigen (PCNA).

We found that 5 pro-apoptotic molecules (FasL, active caspase-8, t-Bid, p53 and Bax) were significantly increased in HRP cultured in intHG conditions, both in normoxia and hypoxia (**Fig. 2**). In particular, FasL increased dramatically in comparison with NG in all experimental conditions, reaching a 39-fold increase in intHG+hypo (p=0.013) (**Fig. 2a**). Active/pro-caspase-8 increase in intHG+hypo reached +70% in comparison with NG (p=0.023), and was significantly higher than NG+hypo and intHG (p=0.003 vs both) (**Fig. 2b**). t-Bid/Bid registered a 3-fold increase in intHG alone (p=0.003) and intHG+hypo (p=0.001 vs NG) (**Fig. 2c**), while p53 increased 4-fold in intHG and 5-fold in intHG+hypo (p=0.001 both vs NG) (**Fig. 2d**). Finally, Bax underwent a 2.1-2.7-fold increase in intHG (p=0.036) and intHG+hypo (p=0.006) vs NG, respectively (**Fig. 2e**).

It is interesting to notice that among these molecules only FasL was increased in stable HG/HG+hypo, to an extent comparable with intHG/intHG+hypo (25-fold increase vs NG, HG: p=0.001, HG+hypo: p=0.015) (**Fig. 2a**). The other mediators were only slightly increased (t-Bid/Bid, p53) (**Fig. 2 c,d**) or even significantly decreased (active/pro-caspase-8, Bax) (**Fig. 2 b,e**). In particular, caspase-8 expression reached -85% in HG (p=0.001) and -50% in HG+hypo (p=0.037 vs NG) **(Fig. 2b).**

Regarding pro-survival markers, BclxL expression was substantially stable in intHG conditions, and the only one that decreased in stable HG and HG+hypo (-75%, p=0.016 vs NG and p=0.017 vs HG) (**Fig. 3a**). We observed a -78% decrease in the Akt phosphorylation (Ser473) in NG+hypo (p=0.003 vs NG) and -49% in intHG+hypo (p=0.018), together with a concomitant increase in HG and HG+hypo (+55%, p=0.001 and + 122%, p=0.022 vs NG, respectively) (**Fig. 3b**). MAPK phosphorylation showed a decrease in intHG+hypo (-40%, p=0.001 vs NG) and an increase in HG+hypo (+54%, p=0.014 vs NG and 0.017 vs NG+hypo) (**Fig. 3c**). Finally, PCNA expression underwent a marked decrease in NG+hypo (-70%, p=0.003), intHG (-29%, p=0.004) and intHG+hypo (-75%, p=0.001 vs NG), and a major increase in stable HG (+110%, p=0.03) and HG+hypo (+120%, p=0.010 vs NG) (**Fig. 3d**).

We did not find any change in the expression of the other pro-apoptotic factors (caspase-9, caspase 3, PUMA, calpain-2) analyzed (data not shown). This was rather surprising regarding caspase-3, which is activated by caspase-8 and considered one of the main executioners of apoptosis. We therefore hypothesized that caspase-8 could act by a direct translocation from the cytoplasm into the nucleus to induce DNA fragmentation and checked our hypothesis by immunofluorescence staining. As shown in **Fig. 4**,

immunolocalization of caspase-8 into the nucleus was evident in HRP cultured in intHG conditions, but not in HG.

Discussion

In this work, we show that hypoxia, alone and combined with intHG, is able to increase HRP apoptosis and decrease their proliferation. Some pro-apoptotic molecules were significantly increased in HRP cultured in intHG with or without hypoxia, concurrently with decreased expression of survival markers, leading to an imbalance in favour to apoptosis. Conversely, in stable HG conditions, most of the pro-apoptotic molecules studied were unchanged or even decreased, and survival molecules increased. Translocation of caspase-8 from the cytoplasm into the nucleus indicates a pivotal role for this molecule in inducing apoptosis.

Loss of pericytes, together with thickening of the basement membrane, is considered one of the early hallmarks of DR. The primary involvement of hyperglycaemia in these events is well-known (Beltramo & Porta 2013), with both direct or mediated actions (Beltramo et al. 2014; Mazzeo et al., 2015), but similar effects of hypoxia have been also recently described (Aplin et al. 2016). Hypoxia, a recurrent event in DR, is due to non-perfusion following closure of capillaries and may cause an increase in VEGF, the major effector of proliferative DR (Nyengaard et al. 2004). In our experimental conditions, hypoxia was able to reduce pericyte proliferation and increase apoptosis, similarly to fluctuating glucose concentrations, while the two factors together concurred in exacerbating this phenomenon. The reduction of the number of pericytes in the capillary wall, due to these concurrent events, determines loss of control on EC proliferation, which, together with VEGF over-expression, may lead to abnormal neo-angiogenesis (Shweiki et al. 1992; Nyengaard et al. 2004).

Cell damage and apoptosis in the diabetic retina have been linked to the activation of death receptors and mitochondrial injury caused by stress stimuli (Valverde et al. 2013). Nevertheless, there is a lack of knowledge regarding the molecular signalling pathways involved in pericyte loss.

Apoptosis, a genetically-determined programmed cell death, occurs physiologically during development and aging to maintain cell homeostasis, but it also works as a defence mechanism against external injuries (Norbury & Hickson 2001). The molecular mechanisms of apoptosis are highly complex, but two main pathways, the extrinsic and the intrinsic, have been described (Elmore 2007; Kiraz et al. 2016). The former involves activation of transmembrane receptors belonging to the superfamily of tumour necrosis factor (TNF) receptors, containing a so-called “death domain” which transmits the death signal from the cell surface to the intracellular pathways (Ashkenazi & Dixit 1998). One of the best characterized ligand-receptor complexes containing the death domain is Fas/FasL (Elmore 2007). The binding of FasL to Fas receptor (FasR) results in the activation of a cascade of intracellular factors which finally leads to the cleavage of procaspase-8 to active caspase-8 (Wajant 2002).

The intrinsic pathway activation involves a series of non-receptor mediated stimuli (such as viral infections, radiation, and also hypoxia), acting directly within the cells (Elmore 2007) and responsible for changes in the inner mitochondrial membrane. This results in opening of the mitochondrial permeability transition pore, loss of the mitochondrial transmembrane potential and release of pro-apoptotic proteins (mainly cytochrome-c) (Saelens et al. 2004). The control and regulation of these apoptotic mitochondrial events is delegated to

members of the Bcl-2 family (Cory & Adams 2002), which can be pro- (for instance, Bax, Bid, Bim, PUMA) or anti-apoptotic (such as Bcl-2 and BclxL), with p53 playing a critical role in their regulation (Schuler & Green 2001).

We therefore investigated the expression of some of the major mediators of these pathways. We found a dramatic over-expression of FasL in all our experimental conditions, reaching its peak when pericytes were grown in intHG plus hypoxia. Moreover, active caspase-8 was significantly increased in intHG plus hypoxia, leading to hypothesize a primary involvement of the extrinsic pathway in the apoptosis of HRP. However, our results also demonstrate, in the same stress conditions (intHG with/without hypoxia), an increase in the expression of Bax, which classically belongs to the intrinsic mitochondria-mediated pathway. We can therefore hypothesize that both pathways may act synergically to induce diabetes-related pericyte apoptosis. This is supported by our finding of over-expression of tBid and p53, both acting as crosstalk mediators between the two pathways: FasL/FasR may cause mitochondrial damage through the caspase-8-mediated cleavage of Bid (Li et al. 1998; Esposti 2002), while p53 involvement has been described in both the intrinsic and extrinsic pathways of apoptosis, and can also be directly activated by caspase-8 (Kiraz et al. 2016).

Regarding the pro-survival factors, our results show substantial stability of BclxL in intHG conditions with/without hypoxia, and decreased phosphorylation of Akt (Ser473) and MAPK, and of PCNA levels. Phosphatidylinositol 3-kinase (PI3K)/Akt pathway is essential for cell survival and growth during development and carcinogenesis. Akt is a kinase inactive in the cytoplasm until it is phosphorylated in Ser473. Activation of Akt promotes cell survival and proliferation, by inhibiting some of the pro-apoptotic Bcl-2 family members (Zhang et al. 2011). PCNA is involved in DNA replication and repair, as it works as an auxiliary protein of DNA polymerases (Prosperi 1997), and its expression is positively regulated by phosphorylated MAPK (Machalińska et al. 2015).

Taken together, our data suggest an imbalance between pro-apoptotic and pro-survival factors in human retinal pericytes cultured in diabetic-like conditions (intermittent HG and hypoxia) and confirm our previous observations of decreased Bcl-2 to Bax ratio in HRP cultured in fluctuating glucose (Beltramo et al. 2009a).

Interestingly, pericytes cultured in stable HG showed a rather different behaviour. In this condition, we only found an increase in FasL levels comparable to intHG condition, since p53 and t-Bid increased to a much lesser extent, and active caspase-8 and Bax levels were even decreased. Concurrently, the cell proliferation marker PCNA and the phosphorylated levels of Akt and MAPK, pro-survival signalling intermediates, were significantly augmented, thus indicating a trend towards increased proliferation, rather than a modulation of the anti-apoptotic BclxL. In fact, BclxL expression is usually stimulated in response to the activation of caspase-8, as a sort of compensatory anti-apoptotic mechanism (Elmore 2007). This diverse behaviour of human pericytes when cultured in intermittent HG as opposed to stable HG is in agreement with our previous observations (Beltramo et al. 2009a; 2009b), showing that these cells, differently from other animal-derived pericytes, are much more sensitive to fluctuating glucose conditions mimicking the situation in diabetic patients, while they seem to develop a sort of passive resistance to stable hyperglycaemic-like conditions.

The extrinsic and intrinsic apoptotic pathways finally converge into the activation of the executioner caspases which, in turn, trigger endonucleases, leading to DNA fragmentation, and proteases, degrading nuclear and

cytoskeletal proteins (Elmore 2007; Kiraz et al. 2016). Caspase-3 is generally considered the most important among the executioner caspases and is activated by the initiator caspases (caspase-8, caspase-9, or caspase-10). However, we could not observe any significant difference in caspase-3 expression in our experimental settings. This could be explained by the fact that our study model mimics the diabetic microenvironment in early DR, while the activation of pro-apoptotic molecules involved in the opening of the mitochondrial permeability transition pore which induces the activation of caspase-3 may occur in later stages. Our finding of a caspase-8 translocation from the cytoplasm into the nucleus in intHG conditions is in agreement with previous observations showing that active caspase-8 may relocate into the nucleus and act directly as an executioner caspase, cleaving PARP-2, a member of the poly(ADP-ribose) polymerase family involved in DNA repair (Benchoua et al. 2002; Arroba et al. 2005) and thus by-passing caspase-3.

In conclusion, diabetic-like conditions are able to stimulate pericyte apoptosis through activation of pro-apoptotic molecules, thus leading to an imbalance between pro-apoptotic and survival signalling pathways. In this work, we show for the first time in our knowledge that both the extrinsic and the intrinsic apoptotic pathways are involved, caspase-8 playing a primary and independent role in triggering apoptosis. Our identification of the intermediates involved could help find new therapeutic approaches for the prevention of DR at early stages.

Acknowledgements

Part of this work has been presented as an abstract at the 25th European Association for the Study of Diabetes – Eye Complications Study Group (EASDec), Torino (Italy), June 26th-28th, 2015 and at the 51st EASD Annual Meeting, Stockholm (Sweden), September 14th-18th, 2015.

This research was supported by the EUROCONDOR project, grant agreement number 278040, funded by the European Commission's Seventh Framework Programme (theme FP7-HEALTH-2011.2.4.3-1). This publication reflects the views only of the Authors, and the European Commission cannot be held responsible for any use which may be made of the information contained therein.

The Authors declare absence of commercial or propriety interest.

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Figure legends

Fig. 1. **HRP proliferation** (**a**) and **apoptosis** (**b**) following 8-day exposure to NG, HG, intHG with/without hypoxia. Mean of 5 experiments ± SD, *= $p<0.05$ vs NG, $= $p<0.05$ vs intHG

Fig. 2. Expression of **pro-apoptotic factors** in HRP following 8-day exposure to NG, HG, intHG with/without hypoxia. **a**) FasL, **b**) ratio active to pro- caspase-8, **c**) ratio tBid to Bid, **d**) p53, **e**) Bax. Densitometric analysis normalized to α-tubulin, mean of 5 experiments ± SD, *= $p<0.05$ vs NG, $=$p<0.05$ vs NG+hypo, § $p<0.05$ vs intHG, #=$p<0.05$ vs HG. **f**) Representative image of one of the Western blots.

Fig. 3. Expression of **pro-survival factors** in HRP following 8-day exposure to NG, HG, intHG with/without hypoxia. **a**) BclxL, **b**) ratio pAKT to AKT, **c**) pMAPK, **d**) PCNA. Densitometric analysis normalized to α-tubulin, mean of 5 experiments ± SD, *= $p<0.05$ vs NG, $=$p<0.05$ vs NG+hypo. **e**) Representative image of one of the Western blots.

Fig. 4. **Translocation of active caspase-8** from HRP cytoplasm into the nucleus, following 8-day exposure to NG, HG, intHG with/without hypoxia. Immunofluorescence with Ab anti-cleaved caspase-8 (*green*). Nuclei are counterstained with DAPI (*blue*). Magnification 200x. Translocation into the nucleus is evident in intHG and intHG+hypo.

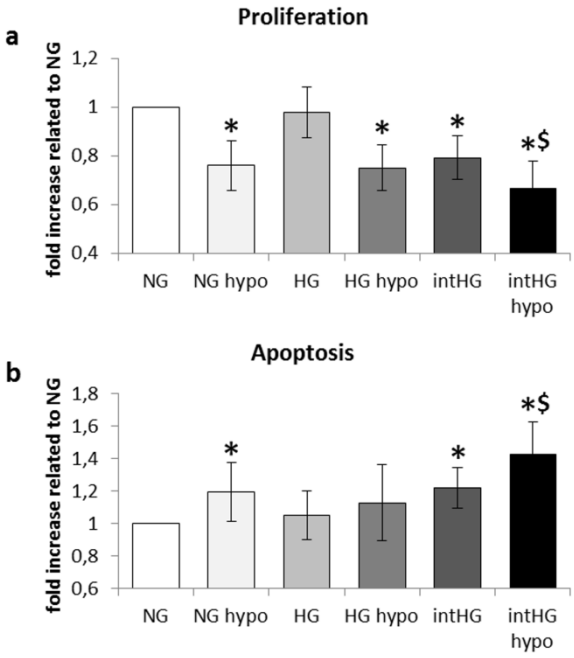
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Figure 1

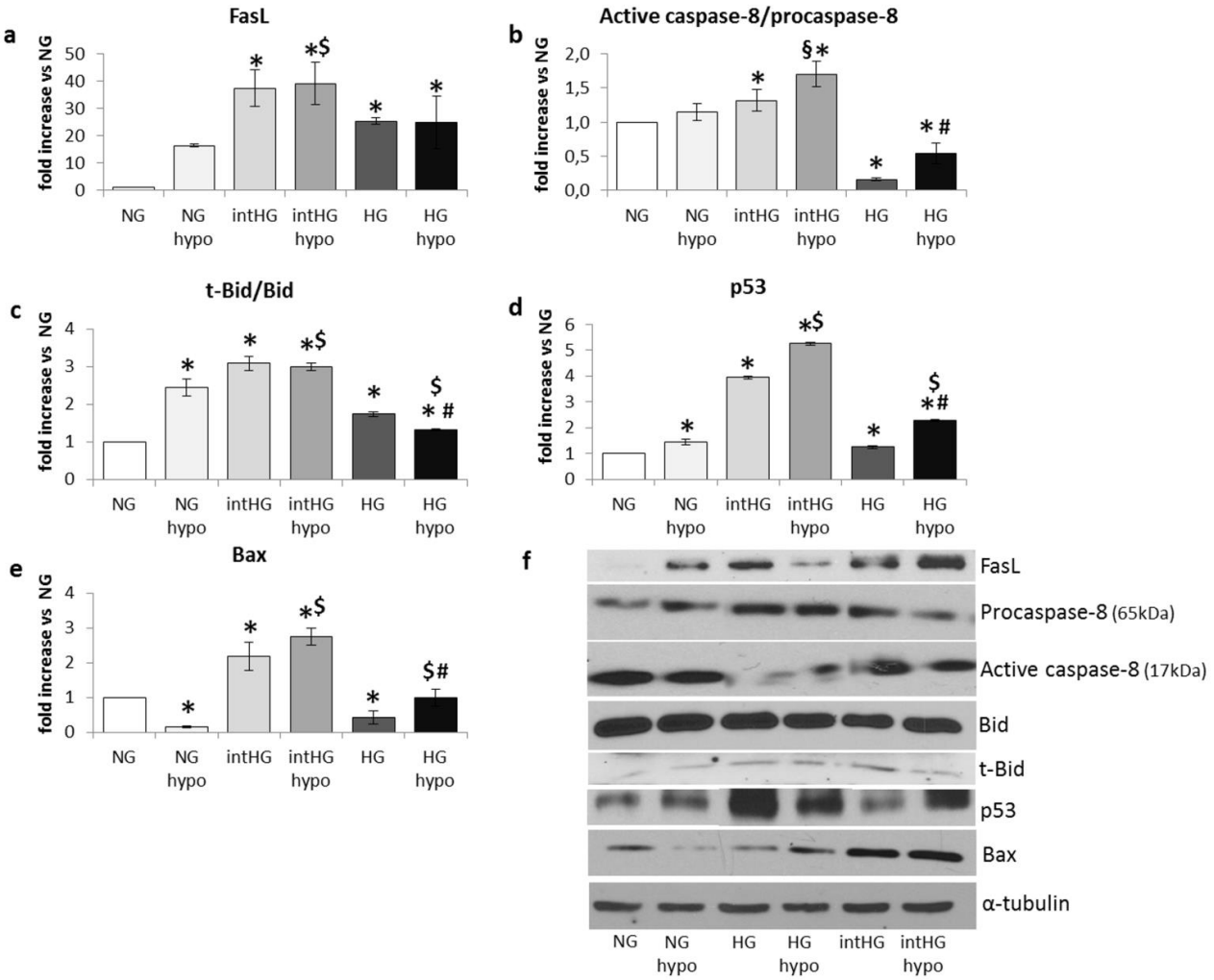
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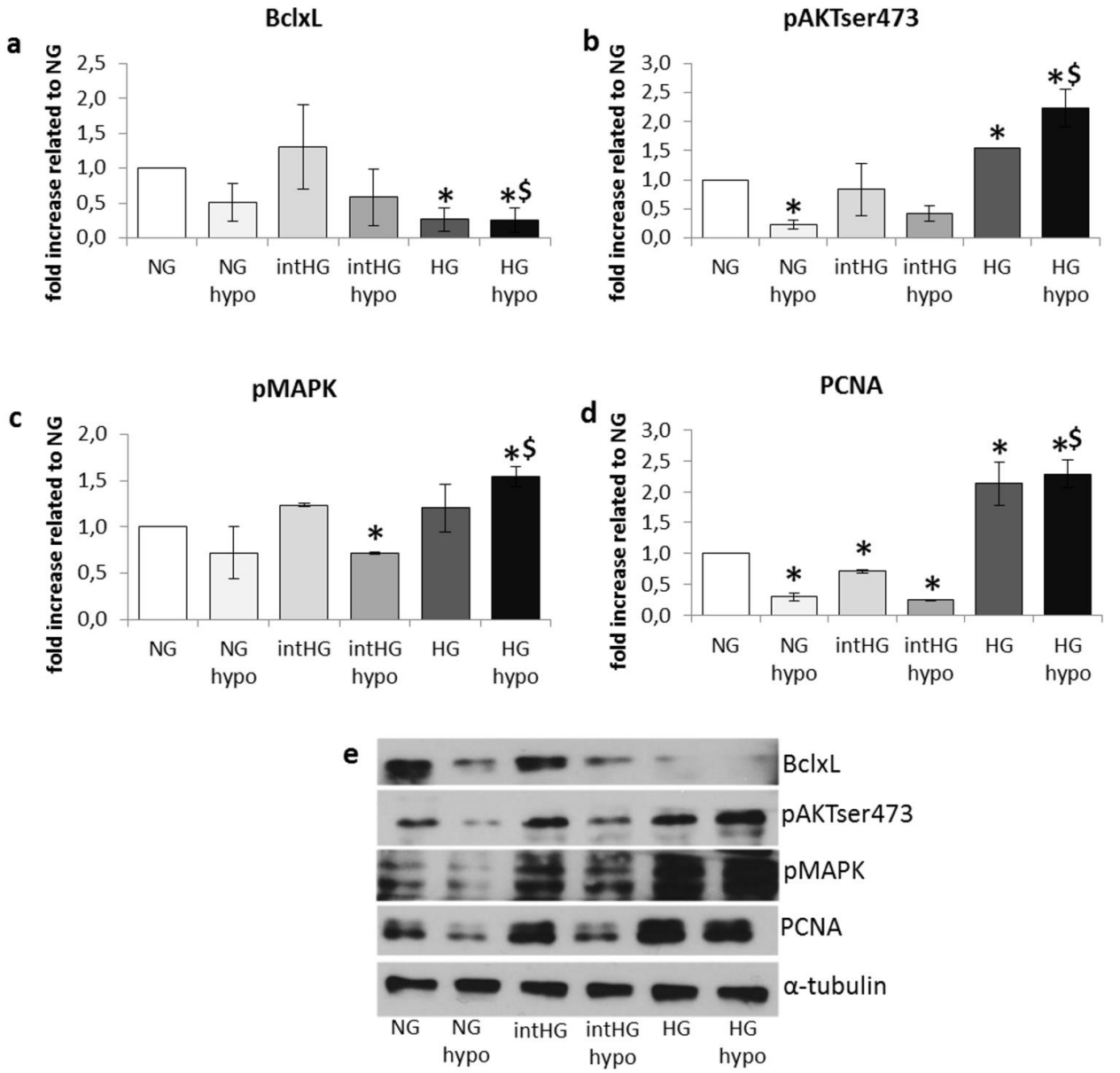
Figure 2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pMAPK
fold increase related to NG
2,0
1,5
1,0
0,5
0,0
*
*$
NG
NG hypo
intHG
intHG hypo
HG
HG hypo
d
PCNA
fold increase related to NG
3,0
2,5
2,0
1,5
1,0
0,5
0,0
*
*
*
*
*$
NG
NG hypo
intHG
intHG hypo
HG
HG hypo
e
BclxL
pAKTser473
pMAPK
PCNA
α-tubulin
NG
NG hypo
intHG
intHG hypo
HG
HG hypo

Figure 3

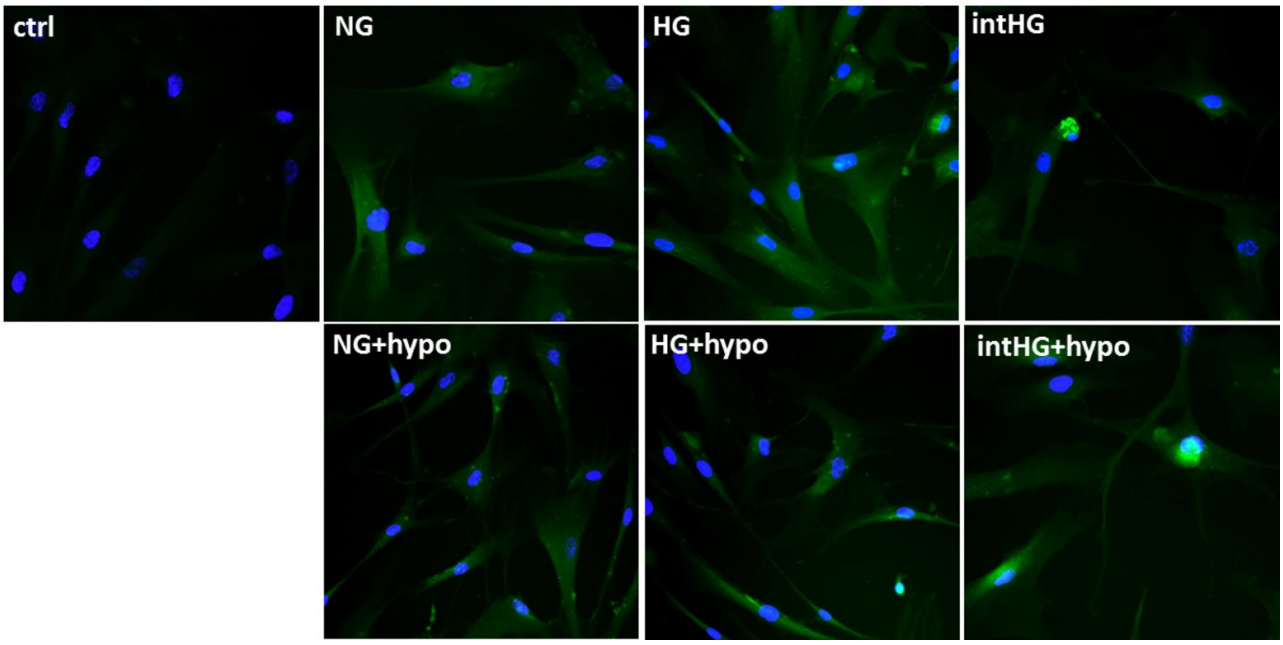

Figure 4