ATTENUATION OF LIVER CANCER DEVELOPMENT BY ORAL GLYCEROL SUPPLEMENTATION IN THE RAT

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Abstract

Purpose: glycerol usage is increasing in food industry for human and animal nutrition. This study analyzed the impact of glycerol metabolism when orally supplemented during the early stage of rat liver carcinogenesis.

Methods: Wistar rats were subjected to a 2-phase model of hepatocarcinogenesis (initiated-promoted, IP group). IP animals also received glycerol by *gavage* (200 mg/Kg body weight, IPGly group).

Results: glycerol treatment reduced the volume of preneoplastic lesions by decreasing the proliferative status of liver foci, increasing the expression of p53 and p21 proteins and reducing the expression of cyclin D1 and cyclin-dependent kinase 1. Besides, apoptosis was enhanced in IPGly animals, given by an increment of Bax/Bcl-2 ratio, Bad and PUMA mitochondrial expression, a concomitant increase in cytochrome c release and caspase-3 activation. Furthermore, hepatic levels of glycerol phosphate and markers of oxidative stress were increased in IPGly rats. Oxidative stress intermediates act as intracellular messengers, inducing p53 activation and changes in JNK and Erk signaling pathways, with JNK activation and Erk inhibition.

Conclusion: the present work provides novel data concerning the preventive actions of glycerol during the development of liver cancer and represents an economically feasible intervention to treat high-risk individuals.

Keywords: proliferation; apoptosis; glycerol; liver preneoplasia; oxidative stress

1 Introduction

Glycerol (propane-1,2,3-triol) is a viscous, colorless and odorless liquid, with sweet taste and completely soluble in water and alcohols. Because of its physicochemical properties glycerol is used in a great number of commercial products including cosmetics, personal care products, pharmaceutical formulations, foods and beverages [1, 2]. Glycerol use is increasing in food industry. Since it gives sweet taste but it does not induce insulin secretion during digestion, glycerol is commonly used as an artificial sweetener, especially in low-fat foods. Glycerol is also used as a thickening agent and a preserving additive in a variety of comestible products [3]. Besides, it has been proposed the use of glycerol as a food supplement in animal diets and it has also been used for rehydration or exercise performance in animals and even humans [4, 5]. Toxicity data for oral glycerol administration indicate that it is safe, with infrequent side effects [6].

In clinical practice, glycerol has been used as an osmotic adjuvant for controlling intracranial pressure [7]. It has also been reported that glycerol inhibits *in vitro* proliferation in various cell types [8] and decreases the cerebral growth of neonatal rabbits [9]. In the liver, glycerol has a potent growth-inhibitory effect *in vivo* during regeneration after partial hepatectomy and *in vitro* in mitogen-induced hepatocyte cultures as well as in a human HCC cell line [10]. However, the mechanisms involved in the antiproliferative actions of glycerol have not been deeply explored.

Hepatocellular carcinoma (HCC) is one of the most lethal tumors worldwide and its prognosis largely depends on tumor stage at the moment of diagnosis. Incidence of HCC has continuously increased over the last years and improved surveillance could be associated with identifiable high-risk patients, like those with chronic liver disease

originated from viral infections, high alcohol consumption or non-alcoholic steatohepatitis, among others [11]. In these patients, liver preneoplastic foci of altered hepatocytes emerge months or years before the diagnosis of HCC [12]. Similar preneoplastic lesions are found in rodents during early stages of liver cancer induced by chemicals [13].

In the present study, we analyzed whether oral administration of glycerol during the early stage of rat liver carcinogenesis is capable of reducing preneoplastic foci development. We also attempt to elucidate the molecular mechanisms associated with this phenomenon.

2 Materials and Methods

2.1 Reagents and Chemicals

Diethylnitrosamine (DEN), 2-acetylaminofluorene (2-AAF) and glycerol were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Anti-pi class of rat glutathione S-transferase (rGST P) was from Stressgen Bioreagents (Ann Arbor, MI, USA). Cy3 fluorescent secondary antibody was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Antibodies against proliferating cell nuclear antigen (PCNA), p53, p21, cyclin D1, cyclin E, cyclin A, cyclin B1, cdk1 (cyclin-dependent kinase 1), cdk2, Bax, Bcl-2, Bad, PUMAα/β, cytochrome c, p-Akt (Ser473), and Akt were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-p-JNK 1/2/3 (Thr183/Tyr185), anti-JNK1/2/3 (against JNK 1 and 2/3 isoforms), anti-p-Erk1/2 (Thr202/Tyr204) anti-Erk1/2 (against Erk 1 and 2 isoforms) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Pierce enhanced chemiluminescence (ECL) Western Blotting Substrate was from Thermo Fisher Scientific

(Rockford, IL, USA). All other chemicals were of the highest grade commercially available.

2.2 Animals and treatment

Experimental protocols were performed according to the NIH "Guide for the Care and Use of Laboratory Animals" (Publication no. 25-28, revised 1996) and approved by the local animal care and use committee (Permission 6060/234, FBioyF, UNR). Adult male Wistar rats were subjected to a 2-phase (initiation-promotion) model of hepatocarcinogenesis, as previously described [14]. All animals received 2 necrogenic doses of DEN (150 mg/Kg body weight, intraperitoneally) 2 weeks apart (initiation phase). The promotion stage began one week after the last injection of DEN; all rats received 2-AAF (20 mg/Kg body weight) by *gavage* 4 consecutive days per week during 3 weeks. Before the start of the initiation-promotion treatment, animals were divided into two goups of six rats each: IP group, animals received the carcinogenic treatment plus a saline solution (glycerol vehicle); and IPGly group, they received the carcinogenic treatment plus 200 mg/Kg body weight glycerol administered by *gavage* once a week, 2 hs before DEN or 2-AAF treatment. A scheme of the experimental protocol is shown in Supplementary Figure 1. Animals were anesthetized with ketamin/ xylazine (100 and 3 mg/Kg body weight, respectively) and sacrificed by exsanguination at the end of the sixth week. Blood samples were collected and livers were removed and processed.

2.3 Serum free glycerol and enzymes activities determination

Serum free glycerol was determined in serum samples using Free Glycerol Determination Kit (Sigma Chemical Co.). Alanine and aspartate aminotransferases (ALT and AST, respectively) and alkaline phosphatase (ALP) were determined spectrophotometrically in fresh serum by commercial kits (Wiener Lab, Rosario, Argentina).

2.4 Immunofluorescence detection and quantitation of rGST P-positive preneoplastic foci

Immunohistochemical detection of rGST P is the chosen method for identification and quantification of preneoplastic foci [15]. Immunofluorescent detection of rGST P-positive foci was performed as previously described [16]. Images were analyzed using ImageJ software (U. S. National Institutes of Health, Bethesda, MD, USA). The number of preneoplastic foci per liver and the percentage of liver occupied by foci were calculated according to the modified Saltykov's method [17].

2.5 PCNA detection and proliferative index determination

Immunohistochemical staining of PCNA protein was performed following the method of Greenwell et al. [18]. Proliferative cells inside the foci and in the surrounding tissue were distinguished by analyzing consecutive section slides stained with anti-rGST P. The PCNA proliferative index was defined as the number of proliferative cells (in G_1, S, G_2 and M phases) per 100 hepatocytes counted in 10 high-power fields. Preneoplastic hepatocytes in each phase of the cell cycle were also determined by a blinded histologist, using specific PCNA staining patterns, as previously described [16, 19]. Data were expressed as percentage of preneoplastic cells in each stage of the cell cycle.

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116 **2.6 Western blot analysis**

117 Whole liver samples were homogenized in 300 mM sucrose with protease and phosphatase
118 inhibitors. Cytosolic, mitochondrial and nuclear extracts were prepared as previously
119 described [14, 16]. Equal amounts of protein were subjected to electrophoresis on 12%
120 SDS-polyacrylamide gels and transferred onto polyvinyl difluoride membranes
121 (PerkinElmer Life Sciences, Boston, MA, USA). Membranes were blocked, washed and
122 incubated overnight at 4 °C with primary antibodies. Finally, membranes were incubated
123 with peroxidase-conjugated secondary antibodies and bands were detected by the ECL
124 detection system and quantified by densitometry using the Gel-Pro Analyzer software
125 (Media Cybernetics, Silver Spring, MD, USA). Equal loading and protein transference
126 were checked by Ponceau S staining of the membranes.

127

128 **2.7 Caspase-3 activity assay**

129 Caspase-3 activity was determined using EnzChek Caspase-3 Assay Kit #1 (Molecular
130 Probes Inc, Eugene, OR, USA), according to the manufacturer's suggestions.

131

132 **2.8 Determination of hepatic glycerol phosphate content**

133 Glycerol phosphate in liver homogenates was enzymatically measured as previously
134 described [20], with slight modifications. First, glycerol phosphate was oxidized by
135 glycerol-3-phosphate oxidase, to generate hydrogen peroxide and dihydroxyacetone
136 phosphate; and second, peroxidase catalyzed the coupling of hydrogen peroxide with 4-

aminophenazone and chlorophenol to produce a quinoneimine dye that can be measured at 540 nm. Glycerol standard solution (Wiener Lab) was used as negative control.

2.9 Lipid peroxidation assay

Lipid peroxidation is considered as an indirect measure of reactive oxygen species (ROS) generation [21]. The amount of aldehydic products generated by lipid peroxidation in liver homogenates was quantified by the thiobarbituric acid reaction according to the method of Ohkawa et al. [22] and measured by high-performance liquid chromatography.

2.10 Liver tissue antioxidant capacity analysis

Reduced (GSH) and oxidized (GSSG) glutathione were determined in total liver homogenates according to the protocol described by Tietze [23], and GSH/GSSG ratio was calculated. Superoxide dismutase (SOD) gel activity assay was based on the method of Donahue et al. [24]. Bands quantification was made by densitometry using the Gel-Pro Analyzer software. Catalase (CAT) activity was determined by monitoring the rate of H_2O_2 decomposition as a function of absorbance decrease at 240 nm [25].

2.11 Determination of protein concentration

Protein concentration was determined by the Lowry method [26], using bovine serum albumin as a standard.

2.12 Statistical analysis

Results were expressed as mean ± SEM. Significance in differences was tested by Student's t-test. Differences were considered significant when the *p* value was < 0.05.

3 Results

3.1 Serum free glycerol levels and hepatic enzymes activities did not change after oral administration of glycerol

Oral administration of glycerol had no effect on serum free glycerol levels measured at the end of the experimental protocol (IP: 0.55±0.06 g/L; IPGly: 0.54±0.04 g/L), as it is rapidly absorbed in the gastrointestinal tract and cleared from blood.

On the other hand, serum markers of liver damage ALT, AST and ALP showed no statistical differences between groups (data not shown).

3.2 Oral administration of glycerol affected the volume of preneoplastic foci

Fig. 1a shows representative images from IP and IPGly groups. Oral administration of 200 mg/Kg body weight glycerol did not induce significant changes in the number of liver foci. However, the percentage of liver occupied by foci significantly decreased in IPGly group as compared to IP animals (Fig. 1b).

3.3 Proliferative status of liver foci was modified by glycerol treatment

Representative images of PCNA staining from the experimental groups are shown in Fig. 2a. Glycerol administration induced a significant decrease of the proliferative index inside

the foci. However, glycerol treatment did not affect the proliferative status of the tissue surrounding the preneoplastic foci (Fig. 2b).

Furthermore, we analyzed the percentages of preneoplastic hepatocytes in each phase of the cell cycle (Fig. 2c). Glycerol administration induced a significant increase in the percentage of cells in G_1 phase of the cell cycle along with a significant decrease in the percentage of cells in M phase.

3.4 Glycerol affected the expression of cell cycle-related proteins

Western blot studies revealed significant increases in the cell cycle-regulatory proteins p53 and p21 in preneoplastic livers of animals treated with glycerol (Fig. 3a and b, respectively). In addition, protein levels of cyclin D1 (Fig. 3c) and cyclin-dependent kinase 1 (cdk1, Fig. 3h) were significantly decreased in IPGly group. Glycerol administration had no effect on cyclins E, A and B neither on cdk2 (Fig. 3d, e, f and g).

3.5 Glycerol administration induced programmed cell death in preneoplastic livers

Oral administration of glycerol significantly enhanced caspase-3 activity (Fig. 4a), which indicated that programmed cell death was occurring. Also, pro-apoptotic Bax levels were increased whereas anti-apoptotic Bcl-2 levels were decreased in liver mitochondrial fractions of IPGly group (Fig. 4b). Accordingly, Bax/Bcl-2 ratio was significantly augmented in animals that received glycerol (Fig. 4c). In addition, mitochondrial levels of pro-apoptotic proteins Bad and PUMA were increased in IPGly animals (Fig. 4d and e). Finally, the release of cytochrome c into the cytosol was increased in IPGly group (Fig. 4f).

3.6 Hepatic levels of glycerol phosphate increased after glycerol treatment

The first stage in hepatic glycerol metabolism is the conversion into glycerol phosphate by glycerol kinase [27]. Fig. 5 shows that hepatic glycerol phosphate levels were increased in IP animals upon oral administration of glycerol.

3.7 Lipid peroxidation and antioxidant capacity were modified by glycerol administration

It has been reported that mitochondrial metabolism of glycerol phosphate generates ROS intermediates [28, 29]. Therefore, we analyzed the amount of thiobarbituric acid reactive substances (TBARS) as a reflection of the hepatic oxidative status. As shown in Fig. 6a, glycerol administration produced a significant increase in TBARS levels compared to IP group. Also, no significant changes in GSH/GSSG ratio were observed between treatments (Fig. 6b). On the other hand, Cu/Zn SOD activity was significantly increased (Fig. 6c) and CAT activity was significantly decreased (Fig. 6d) in IPGly animals. As SOD catalyzes superoxide radical dismutation into O_2 and H_2O_2 and CAT catalyzes the decomposition of H_2O_2 to O_2 and H_2O, it is likely that H_2O_2 is mainly produced during the treatment of IP animals with oral glycerol.

3.8 Glycerol affected JNK1/2/3 and Erk2 activation in preneoplastic livers

Previous studies have shown that pyruvate metabolism produces mitochondrial oxidants release which mediate c-Jun N-terminal kinase (JNK) activation [30]. Since glycerol shares structural and metabolic similarities with pyruvate, we analyzed if oxidative stress generation by glycerol metabolism in IPGly animals was able to activate JNK signaling.

Additionally, we studied extracellular-signal-regulated kinase (Erk) and protein kinase Akt activation, which are critical kinases involved in cell proliferation and apoptosis usually deregulated in HCC [31]. The levels of total and activated (phosphorylated) kinases in liver homogenates were measured by western blot and the phosphorylated/total kinase ratios were calculated. As seen in Fig. 7a, there was a significant increase in the p-JNK/JNK ratio (for the three isoforms) in glycerol-treated animals. In addition, there was a significant diminution in the p-Erk2/Erk2 ratio, with no changes in the activation of Erk1 isoform in IPGly group (Fig. 7b). Finally, p-Akt/Akt ratio showed no differences between the experimental groups (Fig. 7c).

4 Discussion

In the present study we tested the potential antiproliferative effect of oral glycerol supplementation in early liver carcinogenesis and also explored the mechanisms by which glycerol exerts such effect.

The selected dose was based in a previous study in rats which evaluated the effect of oral pure glycerol as a food supplement [4]. We observed that serum markers of liver function did not change in IP animals treated with 200 mg/Kb body weight glycerol, in accordance with the unaffected metabolic parameters previously reported. Furthermore, we used an intermittent regimen of administration, as previously reported for quercetin in our experimental model of liver preneoplasia [16].

The analysis of number and size of proliferative lesions clearly showed that glycerol administration induces a reduction in the development of liver foci, without affecting the number of initiated cells that clonally expand to generate preneoplastic lesions, but

reducing the growth rate of these clones instead. Accordingly, the study of the proliferative status of liver foci indicates that a lower number of hepatocytes are entering into the cell cycle in glycerol-treated rats. Our results show that glycerol action seems to be specific for preneoplastic hepatocytes. Experiments in control (non IP) rats showed that glycerol administration did not affect serum liver damage markers, it kept normal hepatic architecture and it did not affect PCNA staining (data not shown), showing that glycerol exerts its actions in hepatocytes primed to proliferate rather than in quiescent liver cells, in line with previous findings on regenerating rat livers [10].

Induction of p53 results in increased p21 protein levels, a critical regulator of cell cycle arrest [32]. Although we did not deepen the study of the mechanisms involved in p53 and p21 activation, the increased expression of these proteins in liver tissue of IPGly animals support both the antiproliferative and the proapoptotic phenomena observed in this experimental group. The decrease in cyclin D1 protein levels in glycerol-treated rats is in line with the accumulation of preneoplastic cells in G_1 phase. We have also observed that glycerol produces a clear decrease in mitosis, most likely induced by the decrease of cdk1 protein, a fact that does not favor cyclin B/cdk1 complex formation necessary for the cell to enter into the M phase of cell cycle.

Dysregulation of the balance between proliferation and apoptosis defines a pro-tumorigenic basis in hepatocarcinogenesis [33]. Consequently, targeting one or both of these features may result in a reduced tumor development. In this context, increased caspase-3 activity in IPGly animals indicates that apoptosis is enhanced after treatment. Furthermore, glycerol increases mitochondrial Bax/Bcl-2 ratio and Bad and PUMA pro-apoptotic proteins expression, together with the release of cytochrome c into the cytosol [34]. Collectively,

these results support the notion that oral glycerol administration induces apoptosis in preneoplastic livers and that the mitochondria is implicated in this phenomenon. Although apoptosis may be initiated in any phase of the cell cycle, most cells undergo apoptosis primarily in the G1 phase, indicating a direct connection between apoptosis and proliferation. This relationship is explained by the presence of many cell cycle regulators/apoptosis inducers such as p53, operating at the G1/S checkpoint [35]. Consequently, it can be assumed that glycerol induces a cell cycle blockage in order to favor the apoptotic process which would be its ultimate effect to reduce the foci development.

After oral ingestion, glycerol is mainly taken up by the liver and converted into glycerol phosphate by glycerol kinase. Once phosphorylated, it is mostly oxidized by glycerol-3-phosphate dehydrogenase to dihydroxyacetone phosphate [27]. It has been demonstrated that oxidation of glycerol phosphate induces mitochondrial ROS formation, both in normal and in pathophysiological conditions. One of the main ROS generated during glycerol phosphate metabolism is hydrogen peroxide, as demonstrated in isolated mitochondria from different tissues, including hepatic tissue [29, 36]. The study of lipid peroxidation and antioxidant enzymes activities showed that glycerol phosphate metabolism induces production of ROS in our experimental model. Although hepatic levels of hydrogen peroxide were not directly measured, the profile of changes in SOD and CAT activities between the experimental groups supports the hypothesis that this molecule is primarily being produced during glycerol treatment.

It has been established that metabolic hydrogen peroxide functions as a central hub in redox signaling in major processes such as proliferation and cell death [37]. One link between

oxidative stress signaling and proliferation/cell death processes is p53 induction by ROS. Another possible connection is ROS-induced modulation of kinases such as JNK, Erk and Akt. JNK signaling is activated in liver tissue of IPGly animals, supporting the well-established role of ROS-induced JNK signaling in apoptotic cell death [38]. Despite we did not observe any changes in activated Erk1 and Akt levels, Erk2 signaling is inhibited in glycerol-treated rats. In line with this finding, it has been reported that glycerol has a stimulating effect on the phosphatase activity that specifically induces Erk2 inactivation [39]. Moreover, Erk activation is also required for G1/S transition via enhanced cyclin D1 synthesis [40].

A recent study of energy metabolism in HCC shows a depression of glycerol phosphate and other energy metabolites concentrations within the tumor [41]. These data indicate that tumor metabolism turns from mitochondrial oxidation to aerobic glycolysis. Furthermore, based in the present findings, we hypothesize that tumoral cells attempt to avoid glycerol phosphate accumulation as a strategy to evade the effects of this metabolite in their growth rate.

5 Conclusion

Our results provide original data concerning the preventive actions of glycerol during the early development of liver cancer. Our postulated mechanism is schematized in Fig. 8. Briefly, glycerol is taken up by preneoplastic hepatocytes and converted into glycerol phosphate. Then, glycerol phosphate undergoes oxidative metabolism inducing mitochondrial oxidative stress generation. ROS act as intracellular messengers, producing p53 activation and changes in JNK and Erk signaling. These phenomena induce cell cycle

arrest and mitochondrial apoptotic cell death that finally conduct to a reduction of liver lesions. Additional experiments using knockdown and knockout techniques might be useful to confirm the proposed mechanism of action of glycerol in the initial development of liver lesions.

This study is the first one to show a foci volume decreasing role of glycerol in the liver of rats with hepatic preneoplasia. It is interesting to note that despite oral glycerol consumption is innocuous and it is considered an "almost inert" molecule; glycerol exerts its effects in a ROS-dependent manner, leading to cell cycle arrest and increased cell death. The effect of glycerol administration on advanced stages of hepatic carcinogenesis is a mandatory step in the study of glycerol anti-proliferative effects. The results presented in this paper pave the way for a better understanding of natural and risk-free molecules that applied in patients with liver chronic diseases, have the potential to decrease morbidity and improve the quality of life for these patients.

Ethical standards: animal studies were performed according to the NIH "Guide for the Care and Use of Laboratory Animals" (Publication no. 25-28, revised 1996) and approved by the local animal care and use committee (Permission 6060/234, Facultad de Ciencias Bioquímicas y Farmacéuticas, UNR).

Conflict of interest statement: The authors declare that they have no conflict of interest.

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Figure legends

Fig. 1 Effect of oral glycerol administration on number and volume of liver preneoplastic foci. (**a**) Representative images of rGST P-positive preneoplastic foci obtained by confocal microscopy (objective: 10X). (**b**) Changes in number of foci per liver and volume percentage of liver occupied by preneoplastic lesions are represented for IP and IPGly groups. IP: rats with liver preneoplasia; IPGly: IP rats treated with 200 mg/Kg body weight glycerol. Data are expressed as mean ± SEM; n = 6. *p< 0.05 vs. IP

Fig. 2 Effect of oral administration of glycerol on the proliferative status of liver foci. (**a**) Representative images of proliferating cell nuclear antigen (PCNA)-positive cells obtained by optical microscopy (objective: 20X). (**b**) Changes in the proliferative index in the foci and the surrounding tissue. (**c**) Determination of the percentage of preneoplastic hepatocytes in each phase of the cell cycle. IP: rats with liver preneoplasia; IPGly: IP rats treated with 200 mg/Kg body weight glycerol. Data are expressed as mean ± SEM; n = 6. *p< 0.05 vs. IP

Fig. 3 Effect of oral administration of glycerol on the expression of cell cycle-related proteins. Western blot analysis of: (**a**) p53, (**b**) p21, (**c**) cyclin D1, (**d**) cyclin E, (**e**) cyclin A, (**f**) cyclin B1, (**g**) cyclin-dependent kinase 2 (cdk2), and (**h**) cdk1. β-actin was detected as loading control. IP: rats with liver preneoplasia; IPGly: IP rats treated with 200 mg/Kg body weight glycerol. Densitometric analysis was performed and data are expressed as percentage of IP group (arbitrarily considered 100%) and are mean ± SEM; n =6 (**a**, **b**, **c**, **g** and **h**) or 4 (**e** and **f**). *p< 0.05 vs. IP

Fig. 4 Effect of oral administration of glycerol on apoptotic cell death. (**a**) Caspase-3 activity was determined in cytosolic fractions and expressed as percentages, being IP group arbitrarily considered as 100%. (**b**) Mitochondrial levels of pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins were analysed by western blot. (**c**) After densitometric quantitation, Bax/Bcl-2 ratio was calculated, and results were expressed as percentage of IP group (arbitrarily considered as 100%). Mitochondrial levels of pro-apoptotic (**d**) Bad and (**e**) PUMA proteins were also evaluated by western blot. (**f**) Release of cytochrome c was determined by western blot in cytosolic extracts from each experimental group. IP: rats with liver preneoplasia; IPGly: IP rats treated with 200 mg/Kg body weight glycerol. β-actin and prohibitin were probed as loading control in cytosolic and mitochondrial extracts, respectively. Data are mean ± SEM; n= 6 (**a**, **b**, **c** and **f**) or 4 (**d** and **e**). **p*< 0.05 vs. IP

Fig. 5 Analysis of glycerol phosphate hepatic levels. Enzymatic detection of glycerol phosphate in liver homogenates was performed and corrected by protein concentration. IP: rats with liver preneoplasia; IPGly: IP rats treated with 200 mg/Kg body weight glycerol. Results are expressed as percentage of IP group (arbitrarily considered as 100%) and are mean ± SEM; n = 6. **p*< 0.05 vs. IP

Fig. 6 Analysis of lipid peroxidation and liver antioxidant capacity. (**a**) Lipid peroxidation was determined by quantification of the amount of thiobarbituric acid reactive substances (TBARS). (**b**) Determination of reduced glutathione (GSH)/oxidized glutathione (GSSG) ratio in liver homogenates from the experimental groups. Analysis of (**c**) Cu/Zn superoxide

dismutase (SOD) and (**d**) catalase (CAT) activities in total liver homogenates. IP: rats with liver preneoplasia; IPGly: IP rats treated with 200 mg/Kg body weight glycerol. Data are expressed as percentage of IP group and are mean ± SEM; n = 6. *p< 0.05 vs. IP

Fig. 7 Effect of glycerol treatment on activation of JNK, Erk and Akt signalling. Activated (phosphorylated) hepatic protein levels of (**a**) JNK1/2/3, (**b**) Erk1/2 and (**c**) Akt were determined by western blot analysis. Total levels of the kinases were also measured and phosphorylated/total kinase ratios were calculated. . IP: rats with liver preneoplasia; IPGly: IP rats treated with 200 mg/Kg body weight glycerol. Data are expressed as percentage of IP group and are mean ± SEM; n= 4 (**a**) or 6 (**b** and **c**). *p< 0.05 vs. IP

Fig. 8 Scheme showing the postulated mechanisms involved in the preventive action of glycerol in the early development of liver cancer. Inside the hepatocytes, glycerol is converted into glycerol phosphate. Then, glycerol phosphate undergoes oxidative metabolism and generates oxidative stress of mitochondrial origin. Reactive oxygen species (ROS) act as intracellular messengers, producing p53 activation and changes in JNK and Erk signaling activation. These phenomena induce cell cycle arrest and mitochondrial apoptotic cell death that finally conduct to a reduction of liver lesions.

Figure 1

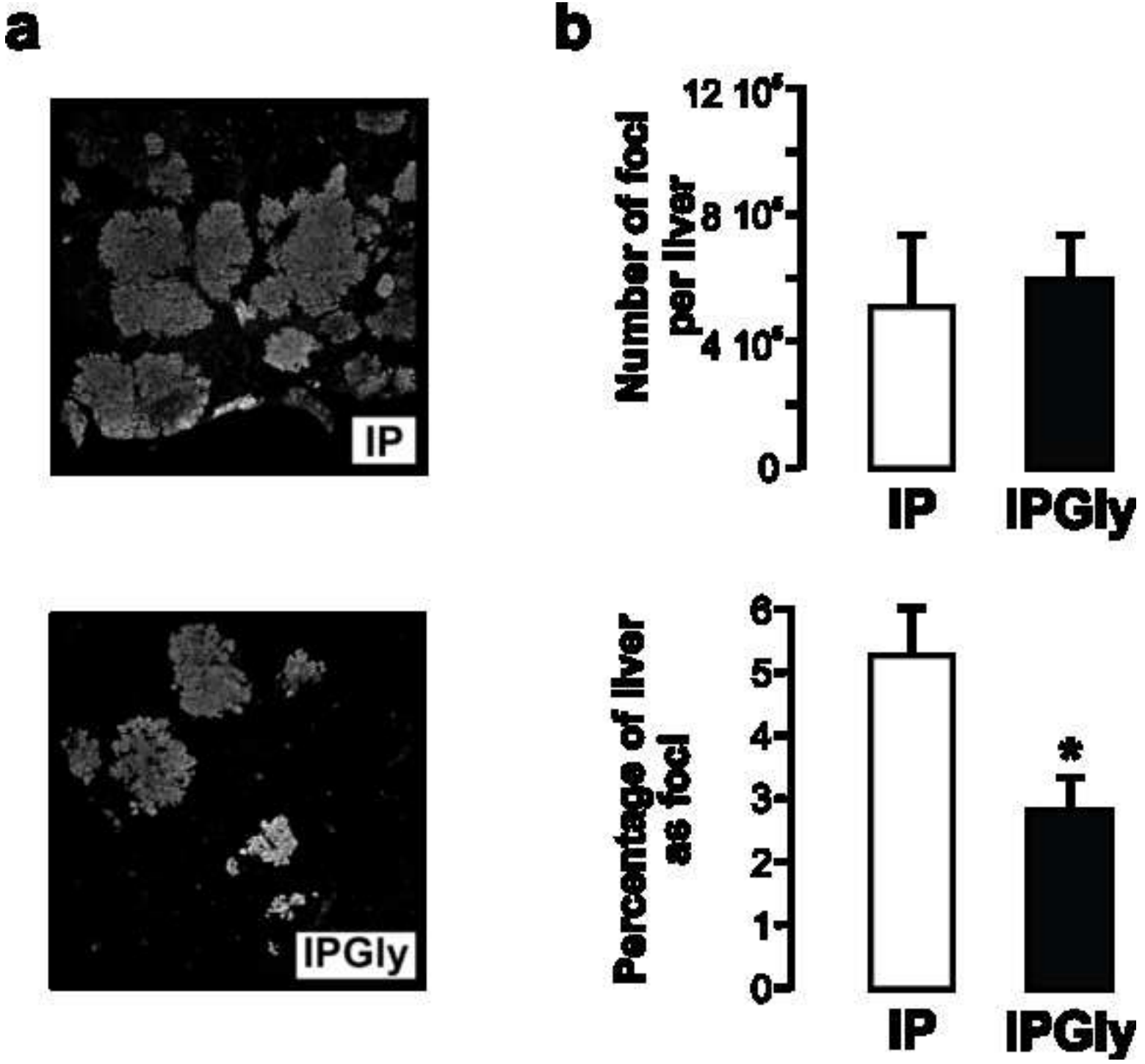

Figure 2

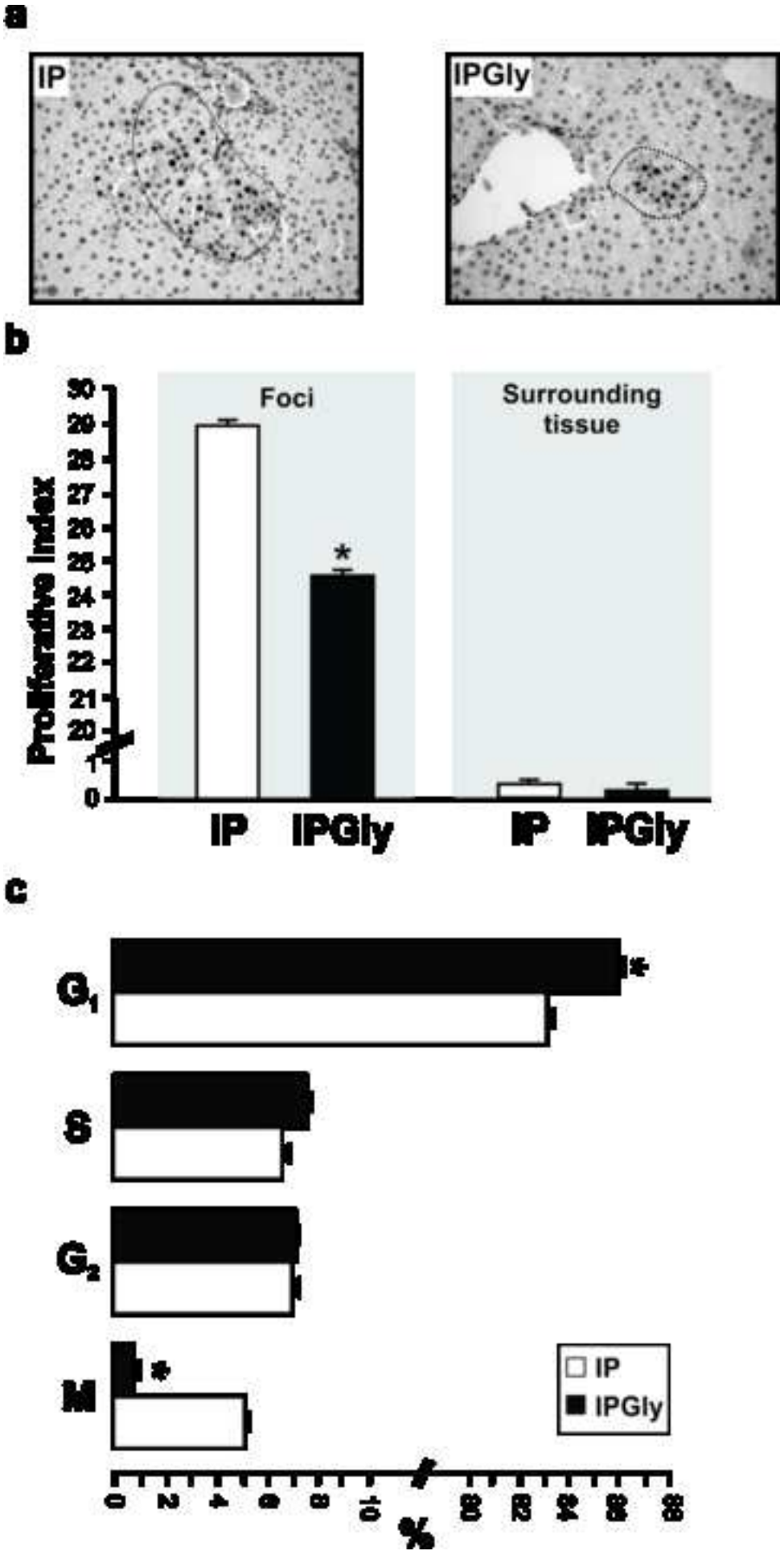

Figure 3

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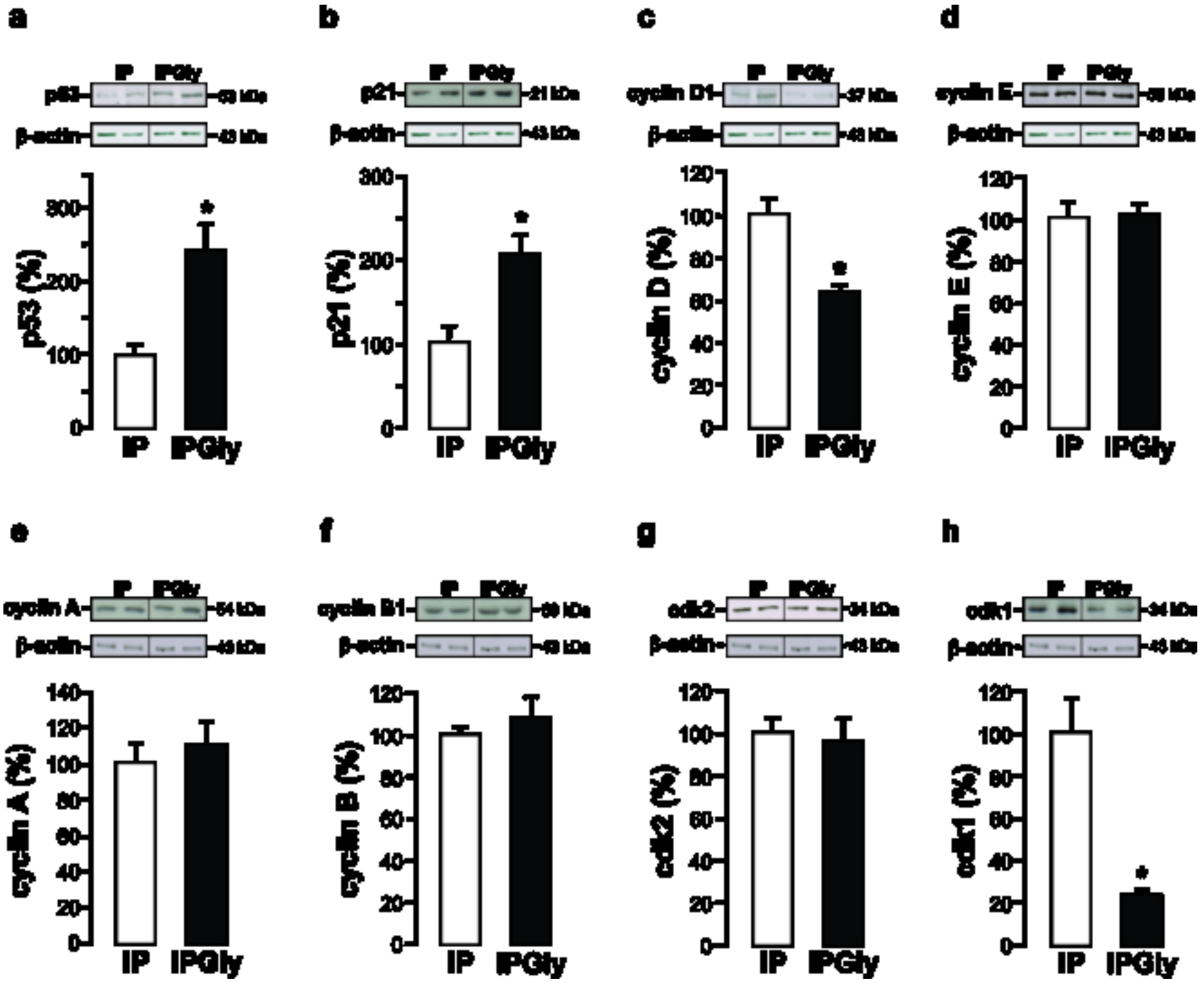

Figure 4

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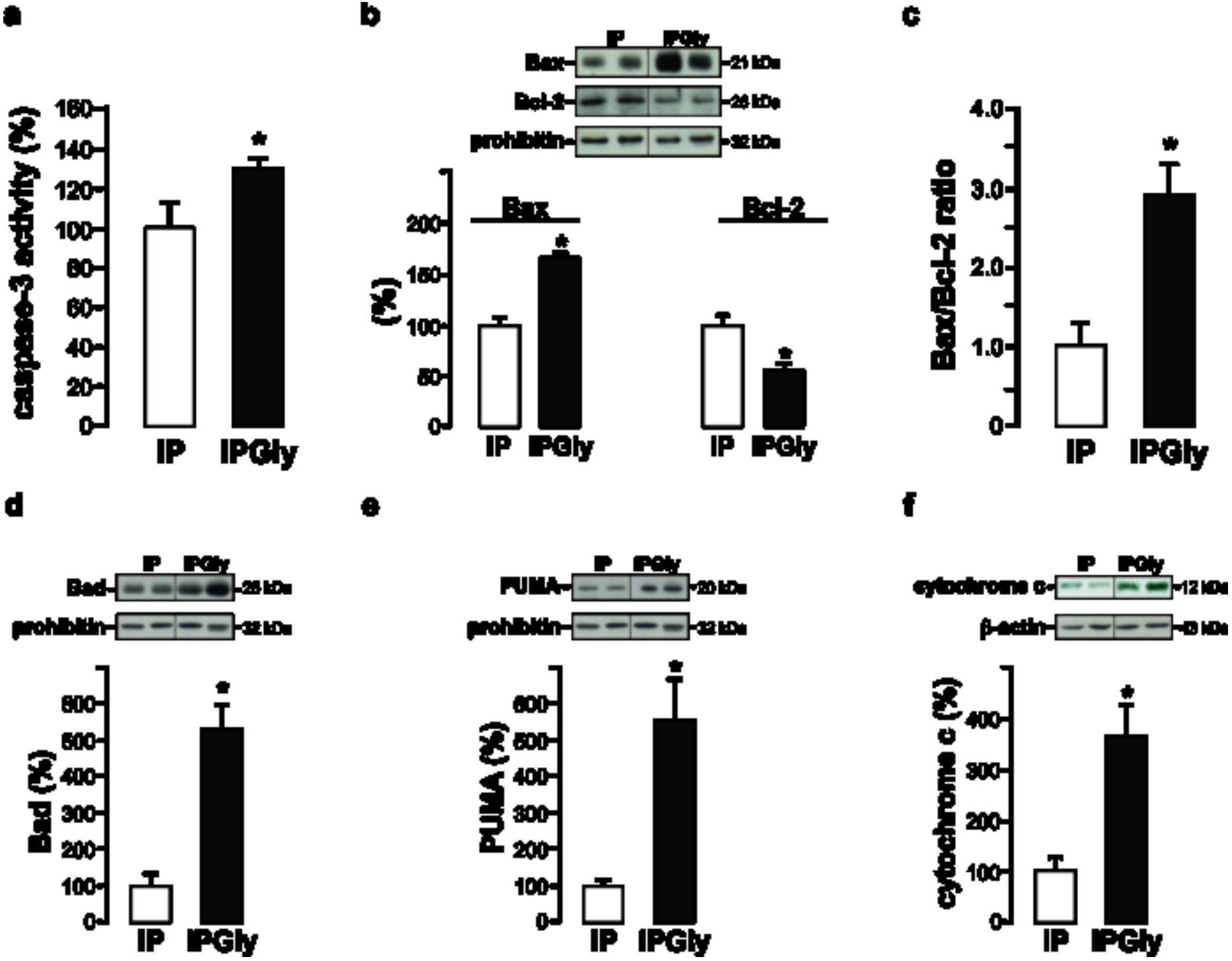

Figure 5

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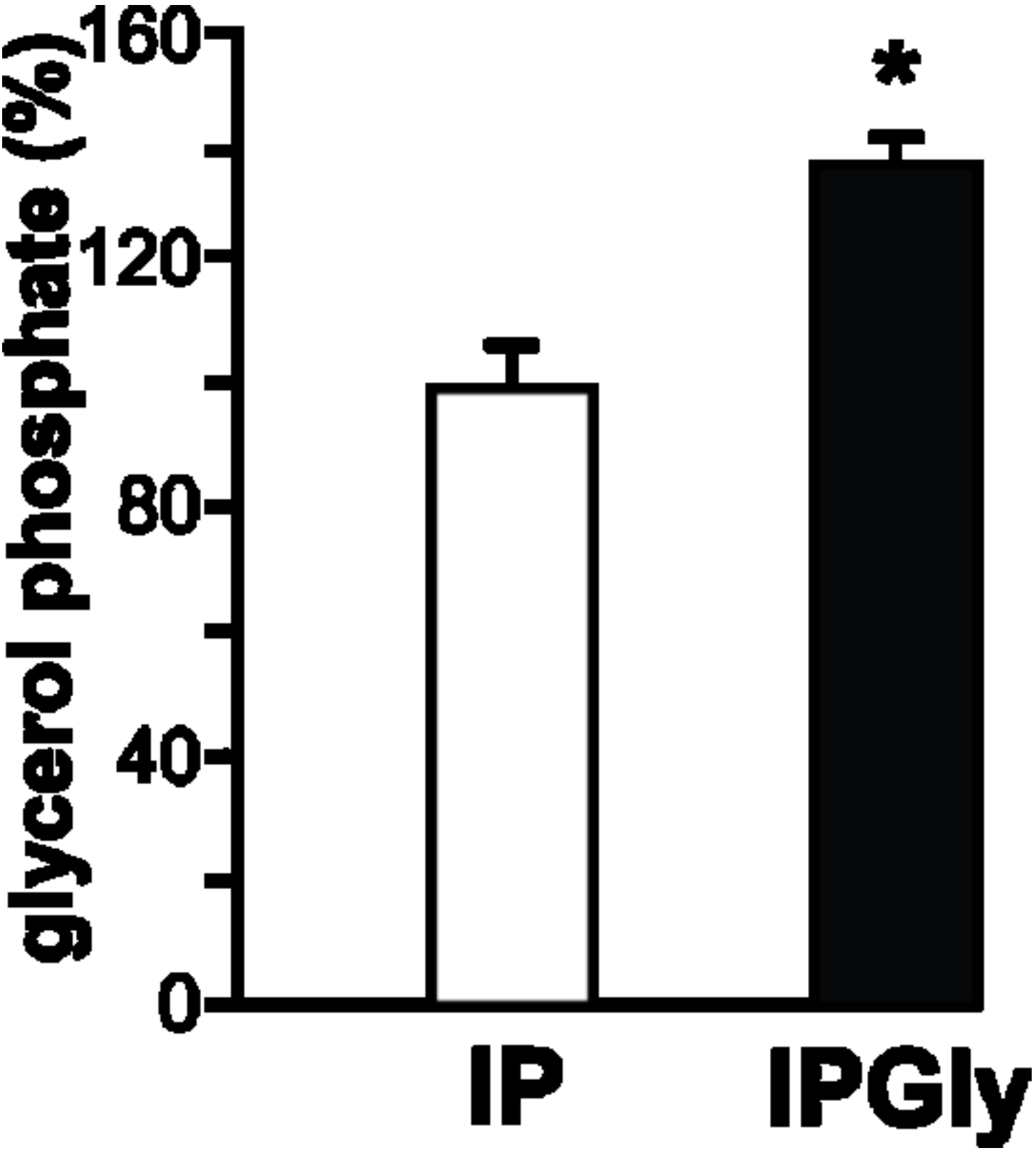

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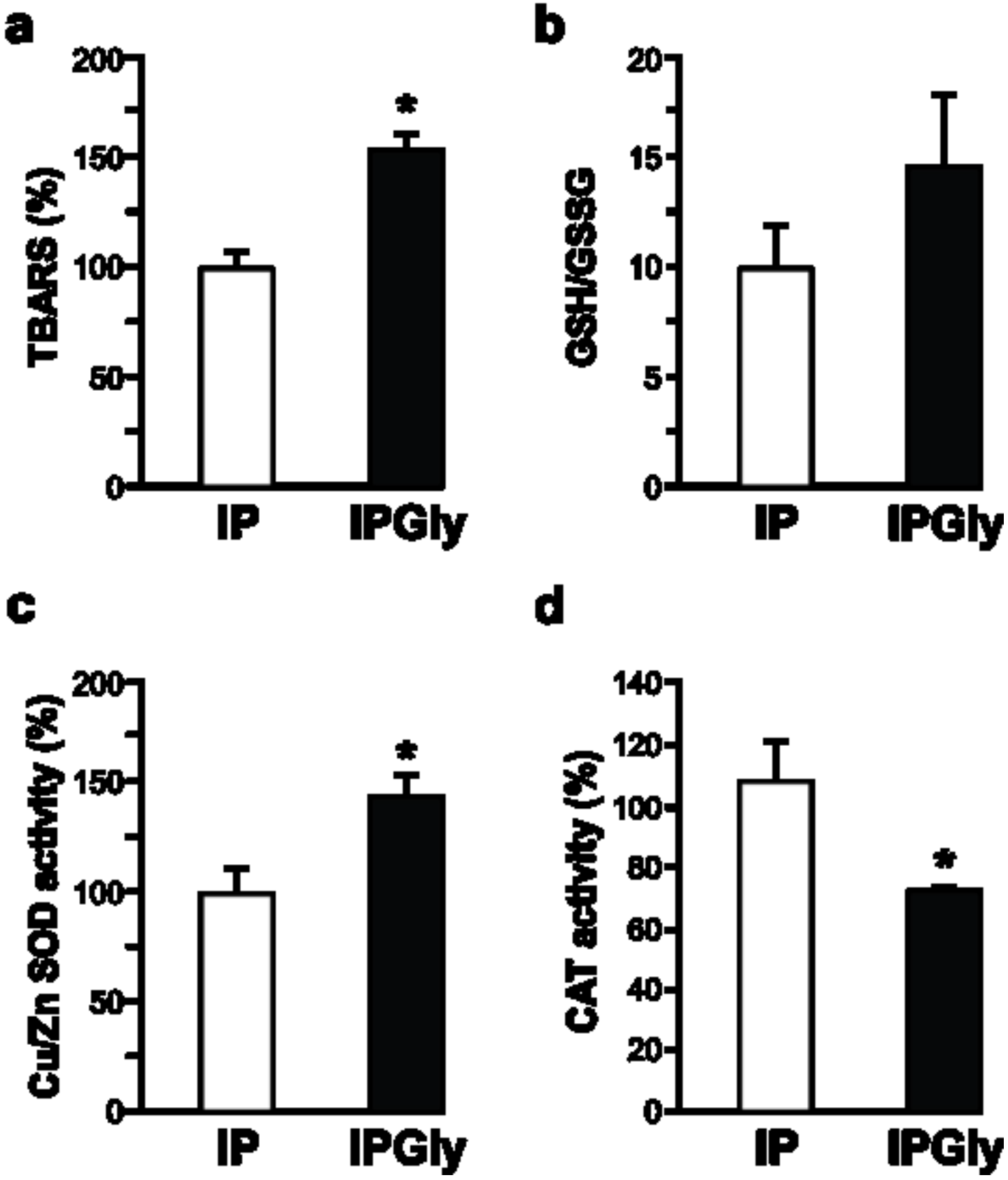

Figure 7

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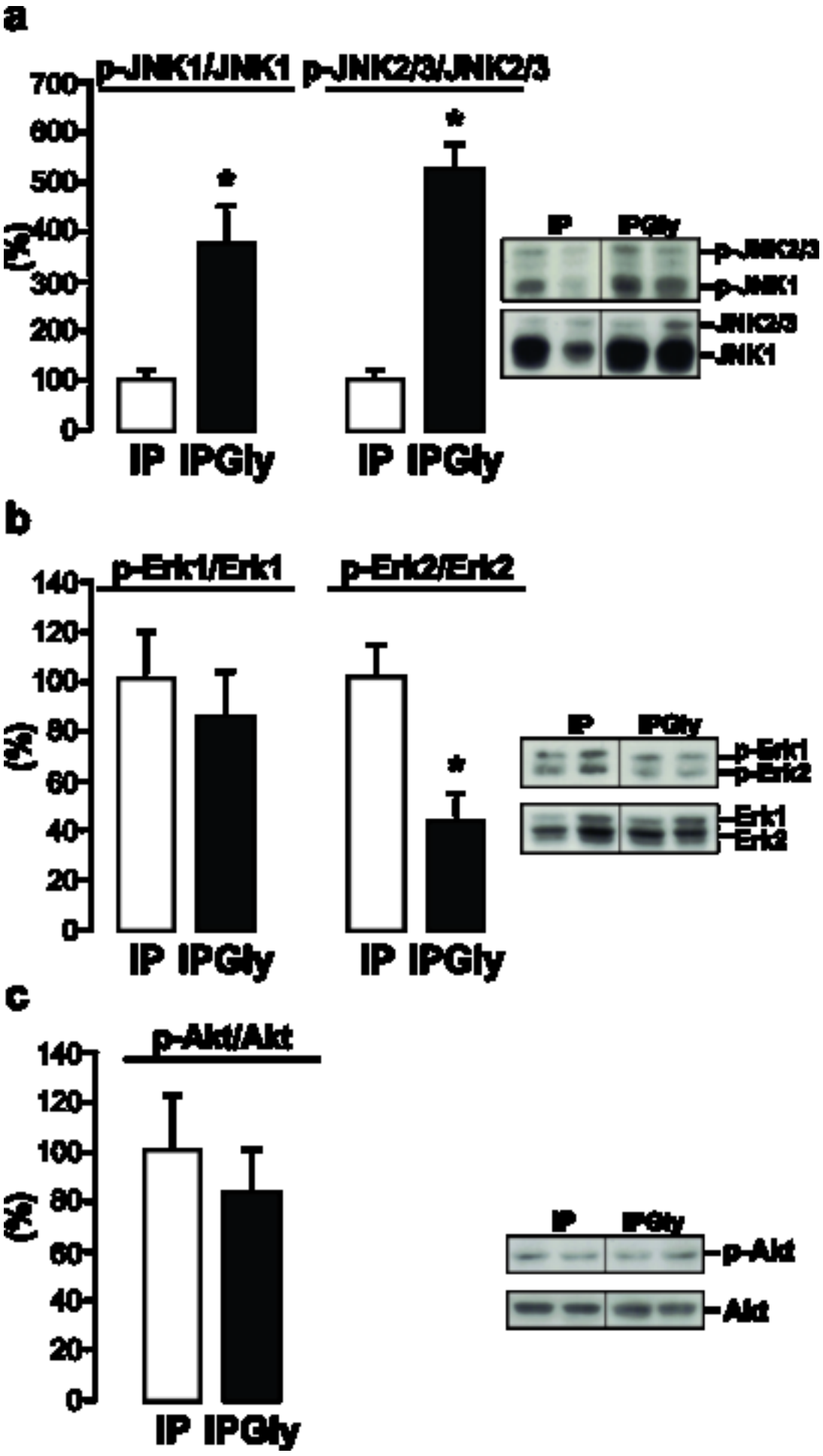

Figure 8

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