Darbepoetin alfa reduces cell death due to radiocontrast media in human renal proximal tubular cells

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ABSTRACT

The hypersialylated erythropoiesis stimulating agent (ESA) darbepoetin alfa was developed for the treatment of anemia, and has also been reported to have other nonerythropoietic effects. This study outlines one such effect against the toxicity of the radiocontrast medium (RCM) sodium diatrizoate (NaD) in human renal proximal tubular (HK-2) cells in vitro. Using a standard cell viability assay, we observed that pre-incubation of HK-2 cells with darbepoetin (at concentrations of 0.25 and 1.0 μg/mL) for 2.5 h prior to addition of NaD (75 mg I/mL, for 2 h) reduced the decrease in cell viability due to the RCM, assayed 22 h after removal of the NaD, whilst maintaining the cells incubated with darbepoetin. Western blot analysis showed that darbepoetin reduced the phosphorylation of c-Jun N-terminal kinases (JNK)1/2 over a period of 1 h incubation with NaD, but did not have an obvious effect on several other targets associated with cell death/survival. However, incubation of HK-2 cells with darbepoetin for a further 22 h after prior exposure to NaD (75 mg I/mL, for 2 h) and subsequent immunoblotting showed that darbepoetin: caused recovery of the activity (phosphorylation) of pro-proliferative/survival signalling molecules, such as Akt (Ser473), STAT (signal transducer and activator of transcription)3 (Tyr705); decreased activation of the pro-apoptotic transcription factor FOXO3a by increasing its phosphorylation at Thr32; decreased phosphorylation (activation) of p38 Mitogen activated protein kinase; and reduced poly(ADP-ribose)/polymerase (PARP)-1 cleavage. In summary, we present here a beneficial nonerythropoietic effect of darbepoetin alfa against radiocontrast-induced toxicity together with modulation of signalling molecules that play a crucial role in determining cell fate.

1. Introduction

The cloning of the human erythropoietin (EPO) gene [1] and the subsequent production of recombinant human EPO (rHuEPO) has allowed the treatment of anaemic patients with chronic kidney disease without the need for blood transfusions and its associated risks [2]. A need for the hormone to have a longer in vivo half-life resulted in the development of another erythropoiesis stimulating agent (ESA), darbepoetin alfa, which differs from rHuEPO in having more N-linked carbohydrate chains [3]. In addition to their use in the treatment of anaemia, ESAs have also been reported to have other non-haematopoietic effects in many different cell types and tissues, including renal cells [4–6]. Such effects have been notably beneficial in certain models of injury, with anti-inflammatory, anti-apoptotic and pro-proliferative properties of ESAs cited as a possible mode of action [3–5,7–9]. Iodinated radiocontrast media have a direct toxic effect on renal cells in vitro [10–14] exerting their effects, at least in part, on modulation of intracellular cell signalling pathways involved in cell death/survival and inflammation [15–18]. It is therefore feasible that ESAs may have a beneficial effect on renal cells treated with...
radiocontrast agents, at least by counteracting the well-known apoptotic and anti-proliferative effects of these agents [19–21]. Indeed, in a rat model of contrast-induced nephropathy, EPO pre-treatment halted the decline in creatinine clearance induced by the radiocontrast agent iohexolamate [22]. However, no data was presented in this study regarding any effects that EPO may have exerted at the molecular level.

Other later studies showed that EPO could attenuate caspase activation in porcine renal epithelial cells subjected to radiocontrast media [23], whilst others have shown that the desialylated form of EPO, asialoerythropoietin, prevented apoptosis of renal tubular cells in vivo and in vitro possibly by mediation of signaling pathways [24]. However, there is no data regarding the effects of the sialylated darbepoetin on renal cells subjected to the toxic effects of RCM. Hence, using the radiocontrast agent sodium diatrizoate and the human renal epithelial tubule (HK-2) cell line, we set out to investigate any beneficial effect of this ESA on the toxic effects of the radiocontrast agent in renal cells and to probe any associated changes in key signalling molecules in these cells.

2. Materials and methods

2.1. Reagents

The radiocontrast agent sodium diatrizoate (denoted also as “NaD” or “diatrizoate” in the manuscript) was obtained from Sigma-Aldrich Co. (St. Louis, MO – USA; product #S4506, having a purity of ≥ 98 %) and was dissolved in RPMI cell culture medium. The final concentration of diatrizoate used was 75 mg iodine/mL when incubated with the cells, this concentration chosen as being physiologically relevant and calculated based on the dosage commonly used in clinical practice, as mentioned in previous studies [25,26]. ARANESP™ (darbepoetin alfa, also referred to as “D” or “darbepoetin” in the manuscript) was a generous gift from Amgen Italia. MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) was obtained from Sigma-Aldrich (St. Louis, MO- USA). Primary antibodies used in the study were as follows: anti-phospho-Akt (Ser 473), code: 9271, Cell Signaling (Bevelry, MA - USA); anti-phospho-p38 (code: 9211, Cell Signaling); anti-phospho-FoxO3a (Thr 32)/anti-phospho-FoxO1 (Thr 24) (code: 9464, Cell Signaling); anti-phospho-STAT (signal transducer and activator of transcription)-3 (Tyr705) (code: 9131, Cell Signaling); anti-PARP (Poly [ADP-ribose] polymerase)-1 (code: 9542 Cell Signaling); anti-phospho JNK (c-jun N-terminal kinase)1/2 (anti-ACTIVE, Promega, code V7931); anti-β-actin (Sigma-Aldrich).

2.2. Cell culture

The HK-2 cell line, an immortalized human renal proximal tubular epithelial cell line from normal adult human kidney, was used and was obtained from the American Type Culture Collection (ATCC® Number: CRL-2190™) and grown in 100 mm culture dishes (Corning®), as previously described [25,27]. In brief, cells were cultured in DMEM containing Glutamax® (Gibco) supplemented with 10 % Fetal Calf Serum and 100 units/mL Penicillin and 100 μg/mL Streptomycin (Sigma) in an atmosphere of 5% CO2 in air at 37 °C, up to a confluence of approximately 90–100 %.

2.3. Cell viability

Cell viability was measured by the ability of viable cells to chemically reduce MTT, as previously reported [25]. Cells were grown in 6-well plates (Corning®) and upon reaching a confluence of approximately 95 %; the cells were serum-starved overnight, after which they were subjected to the appropriate treatment with darbepoetin and NaD. Three series of 6-well plates were prepared cells were incubated with 0.25 or 1.0 μg/mL darbepoetin (final concentration) or with no darbepoetin and left to incubate for 2.5 h; then, sodium diatrizoate was added at a final concentration of 75 mg iodine/mL for 2 h. After 2 h, the media was removed and replaced with fresh serum-free media containing the appropriate concentration of darbepoetin (0, 0.25 μg/mL, 1.0 μg/mL) and the cells cultured for a further 22 h before undergoing the MTT assay. For the MTT assay, cells were washed once with sterile PBS and incubated with 1 mg/mL MTT (in sterile PBS) for 1 h at 37 °C; they were then dissolved in dimethyl sulfoxide (DMSO). Measurements of the coloured product, as a result of MTT chemical reduction, were made at 540 nm using a Beckman DU800 spectrophotometer. Experiments were carried out at least 3 times.

2.4. Sodium dodecyl sulphate polyacrylamide gel electrophoresis and Western blot analysis

For analysis of the effect of darbepoetin on signal transduction molecules in cells incubated with sodium diatrizoate, HK-2 cells were grown to 95–100 % confluence and then serum-starved 18–20 h prior to experimentation. Cells were incubated with darbepoetin (0, 0.25 μg/mL, 1.0 μg/mL) for 2.5 h followed by sodium diatrizoate (75 mg iodine/mL).

For analysis of signaling molecules over a short time course, cells were harvested at 30 and 60 min after the addition of sodium diatrizoate. For the longer time course, the media was removed and replaced with fresh serum-free media containing the appropriate concentration of darbepoetin (0, 0.25 μg/mL, 1.0 μg/mL) and the cells were cultured for a further 22 h before harvesting.

As previously described by us [28], HK-2 cells, at each time point, were washed with cold PBS and then lysed in buffer containing: 50 mM Tris (pH 7.4), 150 mM NaCl, 5 mM Na4P2O7, 100 mM NaF, 2 mM EGTA, 1 mM DTT, 1 mM NaVO4, 1% (v/v) Triton X-100, 2 μM microcystin, 400 μM PMSF, and one protease inhibitor cocktail tablet (Complete Mini™, Roche GmbH, Germany) added for every 10 mL of lysis buffer, as per manufacturers’ instructions. After briefly vortexing, the samples were then centrifuged at 10000 x g for 10 min and the supernatant was retained (lysate). Part of the supernatant was used to determine the protein content and part utilized for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentrations were determined by using a modified Bradford protein assay [29] protocol in order to obtain an equal loading (approximately 30 μg of each sample were loaded).

Protein extracts were resolved by SDS-PAGE and transferred to a nitrocellulose membrane (Hybond C extra, Amersham Biosciences), as previously described [27]. The membrane was incubated for 1 h at room temperature with 5% (w/v) non-fat powdered milk in a “TBS-Tween buffer” (“TBST”: 20 mM Tris and 137 mM NaCl, pH 7.6, containing 0.1 % (v/v) Tween® 20. The primary antibody, diluted in TBST with 5% (w/v) non-fat powdered milk, was then added to the membrane and incubated overnight at 4 °C. The membrane was then washed three times, 5 min each, at room temperature with TBST and incubated for 1 h with a secondary antibody conjugated with horseradish peroxidase (Dako, Agilent), diluted 1:5000 in TBST with 1% (v/v) non-fat powdered milk at room temperature. It was then washed as above (i.e. three times). The secondary antibodies, conjugated with horseradish peroxidase, were detected by incubation of the membrane with an enhanced chemiluminescence reagent [100 mM Tris—HCl pH 8.8, 2.5 mM luminol, 0.4 mM p-convamic acid, 2.9 mM hydrogen peroxide (added immediately before use)] for 1 min. These experiments were carried out at least 3 times.

The bands on the Western blots were quantified by scanning of the radiographic films followed by estimation of the density and area of the bands using Adobe Photoshop and CS2 software.

2.5. Statistics

Statistical analysis was performed using STATA version 14.0 (College Station, TX, USA). Mean percentages of HK-2 cell survival after exposure
to sodium diatrizoate alone (NaD) or after darbepoetin pre-treatment (0.25D and 1.0D refer to pre-treatment with 0.25 μg/mL and 1.0 μg/mL darbepoetin alfa respectively) were reported as mean and Standard Errors (ES). Subgroup differences among the different experimental (NaD, 0.25D, 1D) and control groups were assessed using Analysis of Variance (ANOVA) with Bonferroni adjustment for multiple comparison [30]. A value of < 0.05 was considered significant.

3. Results

3.1. Pre-incubation of HK-2 cells with Darbepoetin results in an increase in cell viability after exposure to sodium diatrizoate

Incubation of HK-2 cells with sodium diatrizoate (NaD) for a period of 2 h, followed by removal of the medium containing NaD, and allowing the cells to incubate for a further 22 h resulted in a statistically significant decrease in cell viability (Fig. 1), as expected from our previous observations [25,31]. Pre-incubation of the cells with darbepoetin at a final concentration of 0.25 and 1.0 μg/mL for a period of 2.5 h prior to exposure to NaD resulted in a statistically significant increase in cell viability as measured 22 h after removal of the NaD (Fig. 1). While pre-incubation with darbepoetin did not fully restore the viable cell level to that of the control group, nonetheless the viable cell levels were significantly higher in both groups that were pre-treated with darbepoetin than in the group treated with NaD only. The increase in viability was also statistically greater at the higher concentration of darbepoetin. Furthermore, the cells that were treated with NaD seemed to be shrunken in appearance compared with control (non-treated) cells, and those that were treated with darbepoetin and diatrizoate had a similar morphology. The resulting loss in cell viability at the chosen NaD concentration (75 μg I/mL) was approximately 30%. From previous findings, under similar experimental conditions, a 50% decrease in cell viability was also statistically greater at the higher concentration of darbepoetin.

The columns indicate the effect of pre-incubation of HK-2 cells with darbepoetin on cell viability after treatment of cells with sodium diatrizoate.

![Graph showing effect of darbepoetin on HK-2 cell viability](image)

**Fig. 1.** Effect of darbepoetin on HK-2 cell viability after treatment with diatrizoate.

The columns indicate the effect of pre-incubation of HK-2 cells with darbepoetin on cell viability after treatment of cells with sodium diatrizoate in serum-free medium.

CON: Untreated HK-2 cells, in serum-free medium.

NaD: HK-2 cells were treated with sodium diatrizoate (75 μg iodine/mL) for 2 h, after which the medium was removed and replaced with fresh serum-free medium. After 22 h, cell viability was determined by the MTT assay, as described in the Materials and Methods section.

0.25D: as for NaD, except the cells were pre-treated with 0.25 μg/mL darbepoetin for 2.5 h prior to treatment with sodium diatrizoate for 2 h.

1.0D: as for NaD, except the cells were pre-treated with 1.0 μg/mL darbepoetin for 2.5 h prior to treatment with sodium diatrizoate for 2 h.

A p value < 0.05 is significant.

3.2. Investigation of changes in signaling molecules upon exposure to sodium diatrizoate for 30 and 60 min in HK-2 cells with/without pre-incubation with darbepoetin

The panels in Fig. 2a show the changes in signaling molecules that are known to play important roles in cell death, survival and proliferation. The phosphorylation (activation) of the mitogen activated protein kinase (MAPK) family members, p38 and JNKs (c-jun N terminal kinases) are both dramatically increased by NaD at 30 and 60 min. A decrease was noted in phospho-p38 at 30 min when pre-incubated with darbepoetin at a concentration of 1 μg/mL, but more dramatic decreases were noted in pJNK1/2, when incubated with the ESA. At 30 min, the decrease in pJNK1 and pJNK2 by both concentrations of darbepoetin are comparable, but at 60 min the decrease in pJNK1 is greater at the higher darbepoetin concentration. As we have previously reported, NaD causes a substantial decrease in pAkt (Ser473), whilst also causing a decrease in pSTAT3 (Tyr705) and pFOXO3a (Thr32) with time [25,32]. Prior incubation with darbepoetin did not have any discernible effect on the decrease in pAkt levels, but accentuated the decrease in pSTAT3 and pFOXO3a levels.

3.3. Changes in signaling molecules in HK-2 cells initially exposed to sodium diatrizoate for 2 h and then incubated for a further 22 h in the presence of darbepoetin

The panels in Fig. 3a show the changes to several signaling molecules including some that were investigated in Fig. 2. It can be seen that 22 h after removal of NaD, the pAkt levels in HK-2 cells treated only with diatrizoate are markedly diminished, but in those cells previously incubated with darbepoetin, a recovery in pAkt levels is observed increasing with the incubation concentration of the ESA. A similar trend was seen for both pFOXO3a and pSTAT3, although the recovery in the levels of these molecules upon pre-incubation with darbepoetin seemingly was not as high as that of pAkt. Observation of the poly(ADP-ribose)polymerase [PARP]-1 enzyme showed a decrease in the levels and evidence of cleavage of this enzyme, as seen by the decrease in the 113 kDa band and appearance of an 89 kDa band (cleavage product) in cells treated with NaD; cells pre-incubated with darbepoetin seemed to have recovered levels of the 113 kDa full length PARP-1 and reduced levels of the cleavage product. Conversely, pre-incubation with darbepoetin caused a decrease in phospho-p38 levels in HK-2 cells treated with diatrizoate compared with those not pre-incubated with the ESA.

4. Discussion

The findings in this study suggest that darbepoetin may have a beneficial effect against the toxic effects of radiocontrast agents on human renal tubular cells. This is an additional non-erythropoietic effect of the molecule to those already reported in kidney and renal cells [5,7,8,33]. With an ever increasing elderly population presenting with many comorbidities, the wide use of contrast media has resulted in evermore increasing cases of CI-AKI and any molecule that may counteract the toxicity of RCM is welcome as therapies to counteract CI-AKI are still scarce [11,13]. The efficacy of natural products against the toxic effects of radiocontrast media have also been investigated [34,35]. A knowledge of the mechanism of action of darbepoetin in this setting may be helpful to allow for future modification of the molecule to improve its efficacy. Several studies in both renal and non-renal cells have reported modulation of signaling pathways by darbepoetin [5,9,36]. Yang et al. [36] observed that darbepoetin could suppress the phosphorylation of the serine/threonine kinases JNK1 and JNK2 in human aortic endothelial cells subjected to TNF-α stimulation over a period of 30 min. We endeavoured to follow this and to extend it further to one hour and found that darbepoetin did indeed reduce the phosphorylation of JNKs induced by NaD (Fig. 2a). This observation is of interest, since the JNK kinases have been implicated in cell death and inflammation [37,38].
and have been implicated in CI-AKI [39]. The phosphorylation status of the JNKs for the extended 22 h period after removal of NaD was not investigated, as we have previously reported that pJNK levels decrease to near basal levels in the long term after NaD treatment [31,32]. In contrast to the JNKs, we did not see a similar reduction in phosphorylation status of the p38 MAPKs over the 60 min time course, confirming a similar observation made by Yang et al. [36]. However, over a longer time period i.e. removal of NaD from the cell medium after 2 h, and incubating the cells for a further 22 h with darbepoetin, a decrease in the phospho-p38 signal was observed when compared with those cells to which darbepoetin was not added. Again, this suggests that darbepoetin could reduce the activation of this kinase that may also play a role in cell death and inflammation [37,40] and lead to an increase in cell viability that we report. In fact, one study reported that N-acetylcysteine amide afforded protection for renal epithelial cells against radiocontrast media-induced apoptosis by blocking p38 kinase activation [41].

Previous findings from our laboratory indicated that dephosphorylation of Akt may be responsible, at least in part, for the loss in cell viability, since transfection with a constitutively active Akt plasmid ameliorated the loss in cell viability due to diatrizoate in vitro [25]. Furthermore, we have also reported that a white grape juice extract could induce Akt phosphorylation in HK-2 cells, and pre-incubation with this extract caused a partial recovery in both the pAkt signal and cell viability after treatment with NaD [28]. Darbepoetin has been shown to activate the PI3K/Akt signalling pathway in cardiomyocytes in vitro and in the heart in vivo, in playing a protective role in autoimmune cardiomyopathy [9]. Observations made in both the kidney and in renal cells in culture have suggested that EPO may also cause phosphorylation of Akt [42,44]. However, results from Fig. 2 show that incubation of HK-2 cells with darbepoetin could not halt or reverse the dephosphorylation of Akt by NaD, by promoting the phosphorylation of the kinase; in fact, whilst we previously observed the phosphorylation of Akt in cultured HK-2 cells by EPO [43] we have not seen the same effect by darbepoetin in the same cells under similar conditions (unpublished observations); such differences may be attributed to the sialic acid residues engineered into darbepoetin alfa to prolong its half-life (up to 22 residues per molecule) that has resulted in a lower affinity of the ESA for the EPO receptor [3]. Nonetheless, as shown in Fig. 3a, incubation of HK-2 cells with darbepoetin for an extended period after removal of the NaD, partially recovered the phosphorylation status of Akt.

The transcription factor FOXO3a is also regulated by phosphorylation, with the phosphorylation site at Thr32 being a substrate for Akt and also for the serum and glucocorticoid-inducible kinase (SGK) -1 [45]. When active, FOXO3a is able to induce apoptosis in part due to its upregulation of the proapoptotic bcl-2 family member Bim and the cyclin dependent kinase inhibitor p27kip1 [46]. We observed that the pFOXO3a (Thr32) levels closely followed those of Akt, with NaD causing

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![Fig. 2. a) Changes in signaling molecules in HK-2 cells exposed to diatrizoate and darbepoetin/diatrizoate.](image-url)

HK-2 cells were incubated with 75 mg I/mL sodium diatrizoate only (designated as NaD) or were pre-treated with 0.25 µg/mL or 1.0 µg/mL darbepoetin for 2.5 h (designated as 0.25D and 1.0D respectively) prior to addition of sodium diatrizoate. At the times shown in the figure (which correspond to the time after addition of sodium diatrizoate), cells were harvested and cell lysates were prepared and subjected to SDS-PAGE and Western blotting, as explained in the MATERIALS and METHODS section.

b) Histograms of the densitometric measurements of the Western blots of phosphorylated p38, JNK1 and JNK2 MAP kinases as represented in Fig. 2a (shown as the mean ± SE). Densitometric measurements of each protein band were normalized to the corresponding actin bands of the same blot. A p value < 0.05 indicating a significant difference was found for the following groups: p-p38: NaD-30 vs 1.0D-30; and 0.25D-30 vs 1.0D-30; p-JNK2: NaD-30 vs 0.25D-30; NaD-30 vs 1.0D-30; NaD-60 vs 1.0D-60. p-JNK1: NaD-30 vs 0.25D-30; NaD-30 vs 1.0D-30; NaD-60 vs 1.0D-60; 0.25D-60 vs 1.0D-60.
Toxicology Reports 8 (2021) 816–821
820

p-FOXO3a: NaD vs 1.0D.

PARP-cp: NaD vs 1.0D.

phosphorylated p38 and Akt kinases, STAT-3, FOXO3a as represented in Fig. 3 a

Further 22 h (in the presence of darbepoetin), after which the cells were har

HK-2 cells were incubated with 75 mgI/mL sodium diatrizoate only for 2 h

darbepoetin.

p-STAT-3: NaD vs 0.25D; NaD vs 1.0D.

PARP-1: NaD vs 0.25D; NaD vs 1.0D; 0.25D vs 1.0D.

-STAT-3 (Tyr705) by NaD after 60 min of incubation, which was not ameliorated by darbepoetin (Fig. 2a). However, incubation for a longer period with darbepoetin after removal of NaD, resulted in some recovery of pSTAT3 (Tyr705) levels (Fig. 3a).

PARP-1 cleavage is a hallmark of apoptosis and has been reported to be due to the action of caspase-3 and caspase-7, giving rise to enzymatically inactive cleavage products with molecular masses of 89 and 24 kDa [48]. We observed this phenomenon in HK-2 cells initially treated with NaD for 2 h and then left to incubate for a further 22 h without the radiocontrast agent (Fig. 3a). However, cells pre-treated with darbe

In summary, darbepoetin has a significant protective effect on renal cell viability after exposure to the radiocontrast agent sodium diatrizoate, and, whilst the data presented is observational, we can propose that a part of the mechanism lies in maintaining the activation of signaling molecules that may aid cell survival/proliferation and diminish the activation of molecules that are involved in cell death and inflammation. Yang et al. observed decreases in pJNK and pNF-kB in human aortic endothelial cells due to darbepoetin and attributed these effects to the sialic acid residues on the darbepoetin molecule [36] since sialic acid has been reported to act as a scavenger of reactive oxygen species (ROS) [49,50]. Whilst the mechanism of toxicity of radiocontrast agents are not fully understood [12], it is believed that one possible mode of action is via the generation of ROS [51], in which case a reduction in their level by a ROS scavenger such as sialic acid would reduce their detrimental effect on cells leading to an increase in their viability. However, we have previously observed that oxidative stress increases phosphorylation of both JNKs and p38 MAPks in HK-2 cells [43,52] and the fact that pJNK, levels are reduced more than p-p38 levels (Fig. 2a) also reported by Yang et al. [36], it seems that this may not be the only mechanism of action of darbepoetin.

In summary, we present evidence for a beneficial nonerythropoietic effect of darbepoetin alfa against radiocontrast-induced toxicity, which may arise in part due to modulation of signalling molecules that play a crucial role in determining cell fate, and future studies that include in vivo models may help to shed light on this.

Author statement

The authors’ contributions were as follows;

M.A. –Conceptualization; supervision; writing-original draft; writing-review and editing.

M.Pf –Formal analysis; writing-review and editing.

T.F. –Investigation.

I.G. –Investigation.

A.P. –Resources.

M.Pe –Investigation.

G.C. –Investigation.

G.D.S. –Funding acquisition; writing-review and editing.

R.S. – Formal analysis; writing-original draft; writing-review and editing.

Fig. 3. a) Changes in signaling molecules in HK-2 cells previously treated with diatrizoate and incubated for an extended period of 22 h in the presence of darbepoetin. HK-2 cells were incubated with 75 mgI/mL sodium diatrizoate only for 2 h (designated as NaD) or with pre-treatment 0.25ug/mL or 1.0ug/mL darbepoetin for 2.5 h (designated as 0.25D and 1.0D respectively), prior to addition of so

a decrease in its level in the short term (up to 1 h), with pre-incubation of cells with darbepoetin not having an effect in reversing this decrease. However, as with pAkt, after removal of the radiocontrast agent and a longer period of incubation (22 h) with darbepoetin, there was some recovery in pFOXO3a levels which may have been due to phosphorylation by active Akt which had also increased in the cells (Fig. 3a).

The transcription factor Signal Transducer and Activator of Transcription 3 (STAT3) is a transcription factor implicated in cell survival and proliferation [47]. Mao et al. also reported the phosphorylation of STAT3 on Ser727 by darbepoetin in rat cardiomyocytes and in rat left ventricular tissue [9]. We observed a dramatic decrease in the levels of pSTAT3 (Tyr705) by NaD after 60 min of incubation, which was not ameliorated by darbepoetin (Fig. 2a). However, incubation for a longer period with darbepoetin after removal of NaD, resulted in some recovery of pSTAT3 (Tyr705) levels (Fig. 3a).

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Declaration of Competing Interest
The authors declare no conflict of interest.

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