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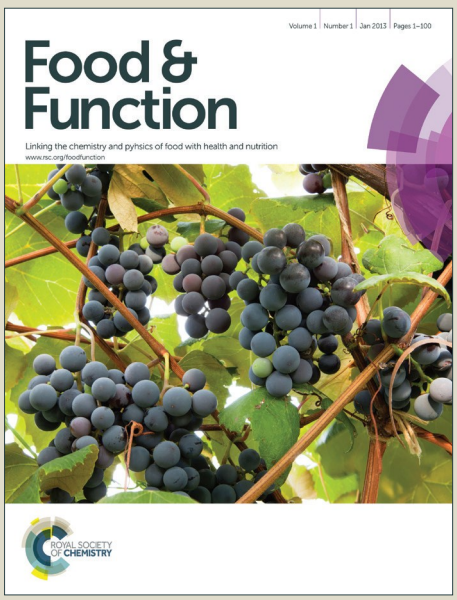

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Hydroxytyrosol decreases the oxidative and nitrosative stress level and promotes angiogenesis through HIF-1 independent mechanisms in renal hypoxic cells

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Abstract

In the kidney, the tissue oxygen tension is comparatively low and renders this organ more prone to hypoxic injury. In fact, hypoxia has a central role in the development and progression of renal disease. The recovery from this situation is dependent depends on the degree to which sublethally damaged cells restore normal function. The master regulator of the hypoxic response is the hypoxia-inducible factor-1 (HIF-1). HIF-1 activity depends on the HIF-1α subunit level which is regulated by oxygen, nitric oxide (NO), reactive oxygen species and mTOR. Given the antioxidant and antinitrosative properties ascribed to hydroxytyrosol (HT), this study evaluates the impact of this olive oil polyphenol on the response to hypoxia in kidney cells. For To that purpose, the human embryonic kidney HEK293T cell line was treated with HT and cultured under sublethal hypoxic conditions. Our results demonstrate that HT treatment decreases both, post-hypoxic reactive oxygen species and NO levels and, consequently, HIF-1α accumulation. However, HT does not affect mTOR activation or the factor inhibiting HIF level but it promotes the expression of angiogenic proteins, suggesting that HT activates an adaptive response to hypoxia in a HIF-1α-independent pathway. In fact, this effect could be ascribed endorsed to the up-regulation of the estrogen-related receptor α. In conclusion, our results propose that in renal hypoxia, HT treatment may be might act as an effective preventive therapeutical approach to decrease the by decreasing stress and to improve improving the adaptive response to this pathological situation.

Keywords: renal hypoxia, hydroxytyrosol, oxidative/nitrosative stress, HIF-1, angiogenesis.

1. Introduction

The reduction of oxygen tension (hypoxia) is associated with a wide range of highly prevalent pathologies such as pulmonary and cardiovascular diseases, diabetic complications, stroke, cancer or inflammation. Particularly in the kidney, the tissue oxygen tension is comparatively low and renders this organ prone to hypoxic injury. This hypoxia susceptibility has a central role in the development and progression of renal vascular disease, acute kidney injury or chronic renal disease and can negatively influence the short- and long-term outcomes of kidney transplantation[1]. Among the complex metabolic reactions occurring during hypoxia, many are related to the formation of reactive oxygen (ROS) and nitrogen (RNS) species. Uncontrolled increases in the concentration of these oxidants play a significant role in the genesis of kidney hypoxic injury. According to the severity of the insult, hypoxic injury to kidney cells can be lethal or sublethal to kidney cells. However, the nature of the recovery response is dependent on the degree to which sublethally damaged cells can restore normal function and promote regeneration[2].

The master regulator of the hypoxic response is the hypoxia-inducible factor (HIF). Three different HIFs factors have been identified, being HIF-1 the key mediator of hypoxic signalling in renal epithelial cells[3]. HIF-1 is a heterodimeric DNA-binding complex, composed of α and β subunits. Because HIF-1β is constitutively expressed, HIF-1 activity depends on the HIF-1α subunit level. Although transcriptional, post-transcriptional and translational mechanisms of regulation have been described, there is no doubt that HIF-1α levels are mainly regulated by post-translational mechanisms, particularly by hydroxylation. Under normoxic conditions, HIF-1α is hydroxylated in Pro-402 and/or Pro-564 by HIF prolyl hydroxylases (PHDs). This post-translational modification triggers protein degradation via the ubiquitin-proteasome system[4]. Because PHDs require oxygen, the reduced oxygen availability in hypoxic conditions inhibits their activity, increasing HIF-1α half-life. PHD activity can also be affected by ROS and nitric oxide (NO), which promote PHD inactivation and HIF-1α accumulation[5]. Moreover, HIF-1α can be regulated independently of PHDs by the phosphatidylinositol-3-kinase/AKT/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway[6]. mTOR is a serine/threonine protein kinase

that phosphorylates p70 ribosomal protein S6 kinase 1 and the eIF-4E binding protein 1, thereby stimulating HIF-1α protein synthesis. Once stabilized, HIF-1α translocates into the nucleus, dimerizes with HIF-1β and, forms the functional HIF-1 transcription factor which and binds to hypoxia-response elements (HRE), inducing to induce the expression of over a hundred genes involved in the adaptive response to hypoxia. HIF-1 transcriptional activity is also regulated in an oxygen dependent way by the hydroxylation of Asn-803 in the C-terminal domain. This reaction, catalyzed by the factor inhibiting HIF (FIH), avoids its interaction with the CBP/p300 coactivator and inhibits HIF-1 transcriptional activity[7].

The Mediterranean diet has been traditionally associated with improved cardiovascular risk profile. Cardiovascular risks factors impair renal function[8] inducing tissue hypoxia. In this sense, recent reports demonstrate that adherence to a Mediterranean diet slows kidney function decline[9,10] and, in fact, this diet has been proposed as a viable alternative to low-protein diet in nephropatic patients[11]. Olive oil is the main source of fat in the Mediterranean diet. Although the beneficial effects of olive oil had been traditionally attributed to its high content of monounsaturated fatty acids, much attention has recently been paid to olive oil minor components. The most representative of those components are phenolic compounds and, particularly, hydroxytyrosol (3,4-dihydroxyphenylethanol; HT). HT penetrates easily into the cells by passive diffusion mechanism[12] and it has been established proven to be a potent antioxidant able to scavenge oxygen and nitrogen free radicals[13,14].

Given the importance of hypoxia and oxidative/nitrosative stress in renal diseases, the key role of both types of stress in HIF-1 regulation and the antioxidant effect of HT, we propose to evaluate if this polyphenol is able to improve the adaptive response to a sublethal hypoxic injury in kidney cells. The results obtained will help to develop new therapeutic interventions against hypoxia in order to decrease the morbidity associated with renal pathologies.

2. Materials and methods

2.1. Cell culture and treatments

Human embryonic kidney HEK293T cells were grown in 10% foetal bovine serum supplemented Dulbecco's modified Eagle's medium (FBS-DMEM, Biochrom^{AG}, Berlin, Germany) at 37ºC in 5% CO_2 and 21% O_2. Cells were pre-treated or not with HT (Extrasynthese, Lyon, France), dissolved in ethanol immediately before use, for 12 h under normoxic conditions (21% O_2). At the end of the incubation time, cells were further cultured for 4h either under normoxic (21% O_2) or hypoxic conditions (1% O_2). Controls were treated with an equal ethanol concentration.

2.2. Cytotoxicity Assay

The cytotoxic effect of HT was evaluated using the sulforhodamine B (SRB) assay as previously described[15]. Briefly, 4 x 10^4 cells/well were plated in 24-well tissue culture plates (Nunc, Rosbilde, Denmark). The plates were incubated for 24 h to allow the cells to adhere. HT was then added to the corresponding wells at a range of concentrations (100, 200, 400, 600 and 800 μM), each concentration being used in at least four replicate wells. After 16 h of treatment, the medium was removed and the cultures were washed with PBS. Cells were fixed at 4^{o}C with 10% trichloroacetic acid (TCA) for 30 min and then washed with tap water to remove TCA. Plates were air dried and stored until use. TCA-fixed cells were stained for 20 min with 0.4% (w/v) SRB (Sigma, St. Louis, Mo, USA) dissolved in 1% acetic acid. At the end of staining period After staining, SRB was removed and cultures were rinsed with 1% acetic acid to eliminate unbound dye. The cultures were air dried and bound dye was solubilized with 10 mM Tris base (pH 10.5). Optical density was read in a plate reader at 492 nm. Cell survival was measured as the percentage of absorbance compared with that obtained in non HT-treated cells.
In addition, the effect of HT and hypoxia on cell viability was also assessed by staining with propidium iodide (PI) and flow cytometric analysis.

2.3. Measurement of intracellular generation of ROS

Intracellular generation of ROS was analyzed using 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Sigma, St. Louis, Mo, USA) as a probe[16]. ROS in the cells oxidize DCFH, yielding highly

fluorescent 2′,7′-dichlorofluorescein (DCF). Briefly, cells were cultured in 24-well plates (5×10^5 cells/well) and treated for 16 h with HT (100 or 200 μM), being cultured during the last 4 h either in normoxic or hypoxic conditions. 30 min before the end of the experiment, cells were washed with Krebs buffer (pH 7.3) and incubated with 10 μM DCFH-DA. Once the incubation was finished, cells were washed three times and DCF fluorescence was measured in a microplate reader (ThermoLabSystem Multiscan Ascent) at an excitation wavelength of 488 nm and emission wavelength of 535 nm.

2.4. NO measurement

Nitric oxide (NO) production was indirectly quantified by determining nitrate/nitrite and S-nitroso compounds (NOx), using an ozone chemiluminescence-based method. To estimate the NOx level, cells of each experimental condition were collected and lysed by 3 freeze-thaw cycles. After centrifugation at 14,000 *g* for 30 min, supernatants were collected and protein was quantified[17]. Samples were deproteinized in deproteinization solution (0.8 N NaOH and 16% $ZnSO_4$). The total amount of NOx in the deproteinized samples was determined by a modification of the procedure described by Braman and Hendrix using the purge system of Sievers Instruments, model NOA 280i[18]. NOx concentrations were calculated by comparison with standard solutions of sodium nitrate. Final NOx values were referred to the total protein concentration in the initial extracts.

2.5. Western blot

For Western blot analysis, equal amounts of denatured total-protein extracts (60 μg for HIF-1α and p-mTOR; 30 μg for Nytrotyrosine and FIH) were loaded and separated in 7.5% (HIF-1α and p-mTOR), 4-12% (Nytrotyrosine) or 10% (FIH) SDS-polyacrylamide gel. Proteins in the gel were transferred to a PVDF membrane (Amersham Pharmacia Biotech., NJ, USA) and then blocked. Polyclonal antibodies to HIF-1α (1/1000, Bethyl Lab, Inc., Montgomery, USA), Nytrotyrosine (1/2000, produced by our group) FIH (1/1000; Santa Cruz Biotecnhology Inc., Santa Cruz, USA) and p-mTOR (1/1000; Cell Signalling, Massachusetts, USA) and a

monoclonal antibody to α-tubulin (Sigma, St. Louis, Mo, USA), as internal control, were used for detection of the respective proteins. Antibody reaction was revealed with chemiluminescence detection procedures according to the manufacturer's recommendations (ECL kit, Amersham Corp., Buckinghamshire, UK).

2.6. Q RT-PCR

Gene expression of AM, VEGF and estrogen-related receptor α (ERRα) were quantitatively assessed by real-time PCR using peptidylprolylisomerase A (PPIA) as the normalizing gene. RNA was isolated using the RNeasyPlus Mini kit (Qiagen, Hilden, Germany) and cDNA was prepared with Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Fermentas Intl., Vilnius, Lithuania). Real-time PCR was performed in a MxPro thermal cycler (Stratagene, California, USA) using SYBR Fast Master Mix (2x) Universal (KAPABiosystems, Massachusetts, USA) with the following gene-specific primers: AM, CTCTGAGTCGTGGGAAGAGG (F) and CCCTGGAAGTTGTTCATGCT (R); VEGF, TTGTACAAGATCCGCAGACG (F) and TCACATCTGCAAGTACGTTCG (R); ERRα, GCTGCCCTGCTGCAACTA (F), GCCTCGTGCAGAGCTTCTC (R); PPIA, TTCATCTGCACTGCCAAGAC (F), TCGAGTTGTCCACAGTCAGC (R). Experiments were performed in triplicate, and the relative quantities of target genes, corrected with the normalizing gene PPIA, were calculated using the Stratagene MxProTM QPCR Software.

2.7. Statistical analysis

Data are expressed as means ± SD. Statistical comparisons between the different experimental groups and their corresponding controls were made with Student's t-test, accepting $p<0.05$ as the level of significance.

3. Results

3.1. HT toxicity in HEK293T cells

The toxicity of HT in HEK293T cells after a 16 h incubation period is shown in Fig. 1. The results obtained indicate that the treatment with up to 600 μM HT does not affect cell growth. However, the highest concentration tested (800 μM) exerts a significant toxic effect ($p<0.05$). Hence, we decided to use 100 and 200 μM HT, concentrations devoid of any negative effect on cell viability.

3.2. Hypoxic treatment induces HIF-1α without affecting cell viability

Sublethal injury is crucial in restoring renal function after a hypoxic episode. Therefore, we next evaluated whether a 4 h hypoxia insult was able to induce a molecular response without affecting cell viability. As shown in figure 2, the hypoxic conditions assayed induced HIF-1α response without affecting cell viability. Moreover, the absence of toxicity of the treatment with at 100 and 200 μM HT in hypoxia was also corroborated by PI staining (Fig. 2B), both in normoxia and hypoxia.

3.3. Effect of HT on post-hypoxic NO production and ROS level

Most of HT effects are usually attributed to an antioxidant effect. The analysis of ROS production by DCFH (Fig. 3A) indicates that HT treatment did not affect the oxidative stress level in normoxia, whereas it decreased hypoxic ROS burst.

We then evaluated whether HT treatment (100 and 200 μM) affected NO level (Fig. 3B). HT did not modify NO levels in normoxia. However, the increase in NO observed in hypoxic cells was significantly reduced by 100 μM HT and completely abolished by 200 μM HT.

As a whole, these results support that this polyphenol protects from oxidative and nitrosative stress under hypoxic situations. In fact, the decreased levels of bulk-nitrated proteins observed in hypoxic HT-treated cells corroborate this hypothesis (Fig. 3C).

3.4. HT decreases HIF-1α expression Effect of HT treatment on HIF-1α expression

NO and ROS are reportedly involved have been previously implicated in HIF-1α stabilization. The difference in the oxidative and nitrosative stress levels observed in HT treated cells

prompted us to determine whether HIF-1α expression was influenced by HT. As shown in Fig. 4, hypoxic HIF-1α induction is abolished by HT treatment.

3.5. mTOR activation is not influenced by HT Effect of HT treatment mTOR activation

The PI3K/AKT/mTOR pathway activation plays an important role in kidney disease progression and is also involved in HIF-1α expression. We then determined whether the phosphorylation of mTOR was modified by HT. Our results (Fig. 5) did not indicate any effect of HT on mTOR activation, suggesting that the PI3K/mTOR pathway is not responsible for the differences observed in HIF-1 expression.

3.6. Effect of HT treatment on angiogenic HIF-1 alpha target genes

We next assessed the expression of two angiogenic HIF-1α target genes (AM and VEGF; Fig. 6). Strikingly, both in normoxic and hypoxic conditions, HT treatment significantly increased the mRNA levels of these two genes.

3.7. FIH expression is not affected by HT treatment Effect of HT treatment on FIH expression

HIF-1 transcriptional activity is inhibited by FIH. We therefore analyzed the effect of HT treatment on FIH levels. The Western-blot results (Fig. 7) demonstrate that the expression of this protein remained invariable in all the experimental groups. The fact that , suggesting that FIH levels are is not affected by HT and suggests the existence of HIF-1 independent mechanisms of regulation involved in the up-regulation of these angiogenic genes.

3.8. HT treatment modulates ERRα expression Effect of HT treatment on ERRα expression

Angiogenic Angiogenesis related genes also respond to ERRα. In an attempt to explain the HIF-1 independent increase in these genes, we next analized analyzed the ARNm mRNA level of this protein. As shown in Fig. 8, HT treatment but not hypoxia induced the expression of ERRα resembling similar to the reported effect of this polyphenol in on the mRNA expression of angiogenic genes.

4. Discussion

Numerous studies have established the relationship between oxidative/nitrosative stress, hypoxia and renal dysfunction[2,19]. Therefore, in the recent years much effort has been devoted dedicated to design treatments to reduce the stress level associated with hypoxia-related renal pathologies while improving recovery. HT, one of the main simple phenols found phenolic compound in olive oil, has been established to be able to scavenge described as a potent scavenger of oxygen and nitrogen free radicals[13,14]; however, its effect in renal hypoxia had not been tested before, and even less let alone in a sublethal hypoxic model in which viable cells are able to trigger molecular pathways leading to an adaptive response. The present study demonstrates that HT treatment is capable of decreasing decreases hypoxic NO and ROS production also in renal cells. NO can be synthesized by the NO synthases (NOS). Although NO from endothelial NOS exert a protective role by maintaining endothelial cells in a quiescent state, high levels of NO, as similar to those observed in our hypoxic conditions, are highly deleterious and promote an inflammatory status. High levels of NO are usually ascribed to the inducible NOS isoform (iNOS) and HT has been proposed to decrease the expression of iNOS this isoform through the impairment of NF-κB activation[20]. Thus, the reduced NO level observed in hypoxic HT-treated cells may reflect a lower iNOS induction, and, consequently, our results support the a protective role of HT treatment against nitrosative stress. HT has also been described to decrease hypoxic oxidative stress in liver and brain ischemic models[21-23]. According to literature, HT exerts its antioxidant effect by directly scavenging ROS or by inducing the activity of antioxidant enzymes such as those of from the GSH system[24,25]. In this sense, our results corroborate this effect in hypoxic renal cells, although further experiments should be performed to assess the particular mechanisms involved.

We next assessed the incidence of this decreased hypoxia-induced stress associated with HT treatment on the expression of HIF-1α, one of the master regulators of the adaptive response to hypoxia. It has been previously published that polyphenols from green tea[26] or red propolis[27] decrease HIF-1 accumulation. Our results are in agreement with those reports since as, under the

same a similar hypoxic stimulus, there is a lower HIF-1α increase induction in HT-treated cells. Both NO and ROS have been described to promote HIF-1α accumulation, although we and others have reported the predominant role of NO rather than ROS on HIF-1α increase[28]. Consequently, it is plausible to hold assume that the decrease in both species associated to HT treatment is could be responsible for the lower HIF-1α accumulation observed.

Apart from oxygen, NO and ROS, HIF-1α expression is also regulated by the PI3K/AKT/mTOR pathway[6]. Moreover, it has been described that mTOR activity can be either activated or inhibited by ROS in a context-dependent manner. Previous data about the effect of HT on this pathway are controversial. In hepatic carcinoma cells this polyphenol has been described to inhibit Akt phosphorylation and mTOR activation[29]. However, HT has also been shown to promote Akt phosphorylation[30,31]. Our present findings in HEK293T provide evidence that HT does not affect mTOR activation either in normoxic or in hypoxic conditions, suggesting that the PI3K/AKT/mTOR pathway is not responsible for the changes in HIF-1α expression and that the effect of HT may vary according to the experimental model studied.

Taking into account that HIF-1 is the key mediator of hypoxic HIF signalling in renal epithelial cells, we have also analyzed the effect of HT on the transcriptional activity of HIF-1 evaluating the expression of two of its typical known target genes, AM and VEGF. In our experimental model HT treatment induced greater mRNA levels of these genes in normoxic and hypoxic conditions. AM and VEGF are key angiogenic factors that induce the formation of new blood vessels from pre-existing ones favouring local blood supply. Therefore, angiogenic factors are cytoprotective in hypoxic renal injury. Moreover, AM suppresses inflammatory cytokines and apoptosis, and has been proposed to ameliorate renal injury via the suppression of NADPH oxidase and the rennin-angiotensin system[32]. In fact, AM levels are highly sensitive markers of kidney disease and could be used as predictive of its prognosis[33]. VEGF is also a pluripotent peptide with cytoprotective and antiapoptotic effects which has been proven to be beneficial in ischemia-induced renal failure[34]. Therefore, the induction of these proteins by HT treatment should be considered as a beneficial effect to counteract hypoxia. Previous literature about the effect of HT on angiogenesis is scarce and point to an antiangiogenic effect of this

polyphenol[29,35]. As none of those studies were performed in renal cells, it should be inferred that the effect of HT is greatly dependent on the cell type and experimental conditions. The only hypoxic model in which that this effect has been studied, was carried out in retina cells using the hypoxia-mimicking agent cobalt chloride ($CoCl_2$) with a subsequent HT-treatment[35]. Apart from inducing HIF-1, $CoCl_2$ affects other important enzymes and pathways, so hence its effects do not strictly reproduce those of a hypoxic situation. On the contrary In our study, we have used a hypoxic model of sublethal damage in renal cells with a previous treatment of HT. ConsequentlyAs a result, we have been able to evaluated the effect of a preventive intervention in cells that can trigger an adaptive response, which could explain the disagreement in the results.

However, the increase in VEGF and AM could not be attributed to a higher HIF-1α accumulation, because the levels of this protein were lower in hypoxic HT-treated cells than in hypoxic non-treated cells. HIF-1 transcriptional activity is regulated by FIH[7]. Therefore, we speculated that a lower expression of FIH could account for the higher transcriptional activity of HIF-1 observed in HT-treated cells. However, no effect of HT on FIH level was detected, suggesting that HT induces the expression of AM and VEGF through HIF-1 independent mechanisms.

A HIF-1 independent mechanism of inducing angiogenesis involves the estrogen-related receptors (ERRs) pathway[36,37]. Angiogenesis has also been shown to be induced by estrogen-related receptors (ERRs) in a HIF-1 independent pathway36,37. ERRs are a family of constitutively active orphan nuclear hormone receptors that dot not require activation with any agonist[38]. Particularly, ERRα, one of the first identified orphan nuclear receptors, is expressed in most tissues at moderate to high levels and, particularly, in organs with high metabolic needs such as kidneys[39]. ERRα recognizes the consensus DNA sequence AAGGTCA. This ERRα response element was initially described in several genes regulating cellular energy metabolism[40]. Moreover, Arany et al. also described the existence of these ERRα-sites both in the promoter and in a conserved enhancer in the first intron of the VEGF[36]. Since then, a number of studies have demonstrated the pro-angiogenic role of ERRα through the direct

transcriptional regulation angiogenic genes[41-43]. In fact, apart from angiogenesis, ERRα activation induces genes with roles in lipid transport, oxidative phosphorylation, fatty acid oxidation, tricarboxylic acid cycle, mitochondrial biogenesis, or oxidative stress defense[40-42]. Our results demonstrate that ERRα expression is not affected by hypoxia. Nevertheless, HT-treatment induces a significant increase in ERRα mRNA both in normoxic and hypoxic conditions, which could explain the higher level of angiogenic genes described above. The potential estrogenic effect of extra virgin olive oil polyphenols has been recently suggested in the literature[4344] but, to our knowledge, this is the first report that supports the induction of ERRα in response to HT.

5. Conclusion

Although further *in vivo* experiments should be done, our results indicate that in renal hypoxia HT decreases the nitrosative and oxidative stress level and induces angiogenesis through HIF-1 independent mechanisms (Fig. 9), suggesting that it may be a plausible preventive therapeutical approach to decrease the stress and to improve the adaptive response to this pathological situation. HT treatment significantly decreased the nitrosative and oxidative stress level associated to renal hypoxia. This effect resulted in a lower HIF-1α accumulation although, unexpectedly, an increase in the response of the angiogenic genes was observed. We propose that this pro-angiogenic action of HT is mediated by a HIF-1 independent mechanism that involves the up-regulation of ERRα (Fig. 9). Although further *in vivo* experiments should be performed, these findings suggest that HT may be a plausible preventive therapeutical approach to improve the adaptive response to renal hypoxia.

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Figure captions

Fig. 1. Effect of HT on HEK293T cell proliferation survival. Values represent the mean±SD from three independent experiments. Statistically significant differences with the corresponding non-treated cells:* $p<0.05$.

Fig. 2. Development of a hypoxic model of sublethal damage. (A) Effect of the experimental hypoxic model on HIF-α induction. Representative immunoblot of HIF-1α expression in normoxic and hypoxic cells. α-tubulin (α-Tub) immunodetection was included as a protein-loading control. (B) Effect of the experimental hypoxic model and HT treatment (100 and 200 μM) on cell viability measured by PI staining. Values represent the mean±SD from three independent experiments.

Fig. 3. (A) Effect of HT treatment and hypoxia on the oxidative stress level measured by DCF fluorescence. (B) Effect of HT treatment and hypoxia on NO levels (μmol/mg of protein). (C) Effect of HT treatment on hypoxic nytrotyrosine level. Values represent the mean±SD from three independent experiments. Statistically significant differences with the corresponding non-treated normoxic cells: * $p<0.05$, *** $p<0.001$. Statistically significant differences with the corresponding non-treated hypoxic cells: $^{a}p<0.05$, $^{aaa}p<0.001$.

Fig. 4. Effect of HT on HIF-1α stabilization. Representative immunoblot of HIF-1α expression in normoxic and hypoxic cells. α-tubulin (α-Tub) immunodetection was included as a protein-loading control. Values represent the mean±SD relative to α-Tub from three independent experiments. Statistically significant differences with the corresponding non-treated normoxic cells: * $p<0.05$. Statistically significant differences with the corresponding non-treated hypoxic cells: $^{a}p<0.05$.

Fig. 5. Western-blot analysis of the effect of HT on p-mTOR levels. Representative immunoblot of p-mTOR expression in normoxic and hypoxic cells. α-tubulin (α-Tub) immunodetection was

included as a protein-loading control. Values represent the mean±SD relative to α-Tub from three independent experiments.

Fig. 6. Effect of HT treatment on the mRNA expression of AM and VEGF. The results are expressed as mRNA expression relative to normoxic non HT-treated cells after normalization against PPIA. Values represent the mean±SD from three independent experiments. Statistically significant differences with the corresponding non-treated normoxic cells: ** $p<0.01$, *** $p<0.001$. Statistically significant differences with the corresponding non-treated hypoxic cells: $^{aa}p<0.01$, $^{aaa}p<0.001$.

Fig. 7. Western-blot analysis of the effect of HT on FIH levels Representative immunoblot of FIH expression in normoxic and hypoxic cells. α-tubulin (α-Tub) immunodetection was included as a protein-loading control. Values represent the mean±SD relative to α-Tub from three independent experiments.

Fig. 8. Effect of HT on the transcription of ERRα. The results are expressed as mRNA expression relative to normoxic non HT-treated cells after normalization against PPIA. Values represent the mean±SD from three independent experiments. Statistically significant differences with the corresponding non-treated normoxic cells: ** $p<0.01$, *** $p<0.001$. Statistically significant differences with the corresponding non-treated hypoxic cells: $^{aa}p<0.01$.

Fig. 9. Proposed model of action of HT-treatment in the hypoxic response of sublethally damaged renal cells. In the absence of HT hypoxia raises both NO and ROS levels promoting HIF-1α accumulation and HIF-1 transcriptional activity. HT-treatment dampens hypoxia-associated stress increase, inhibiting HIF-1α post-hypoxic accumulation. However, its effect on ERRα expression further enhances an angiogenic response.

Figure 1.

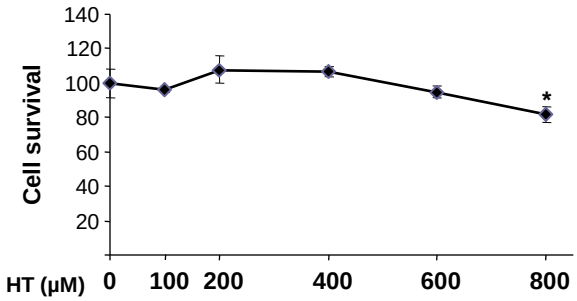
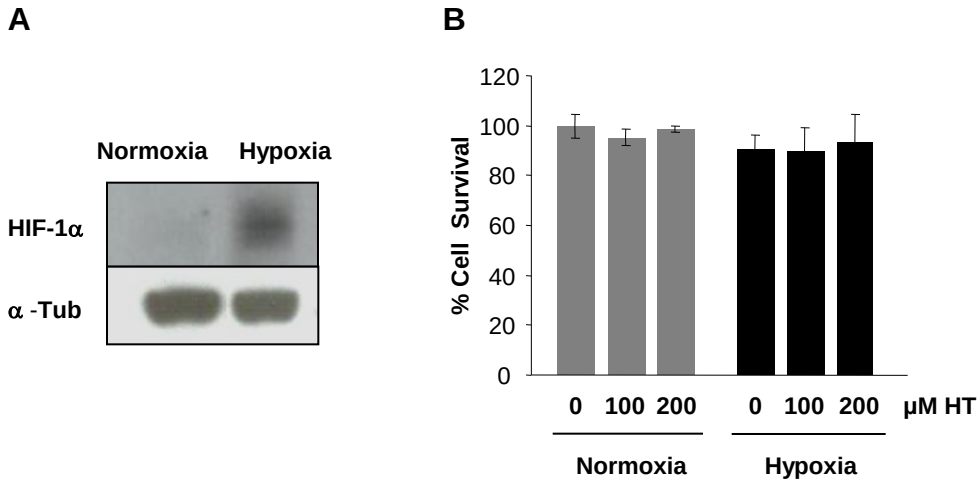


Figure 2.



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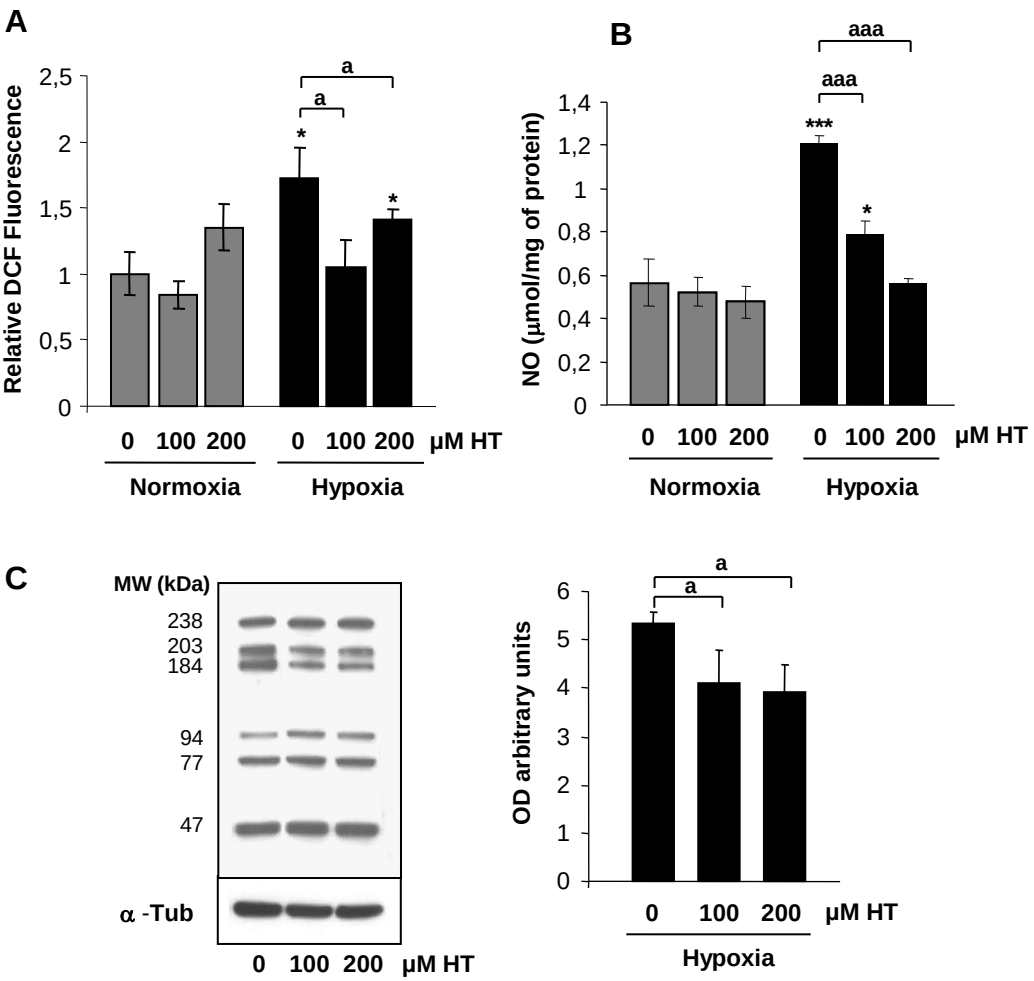

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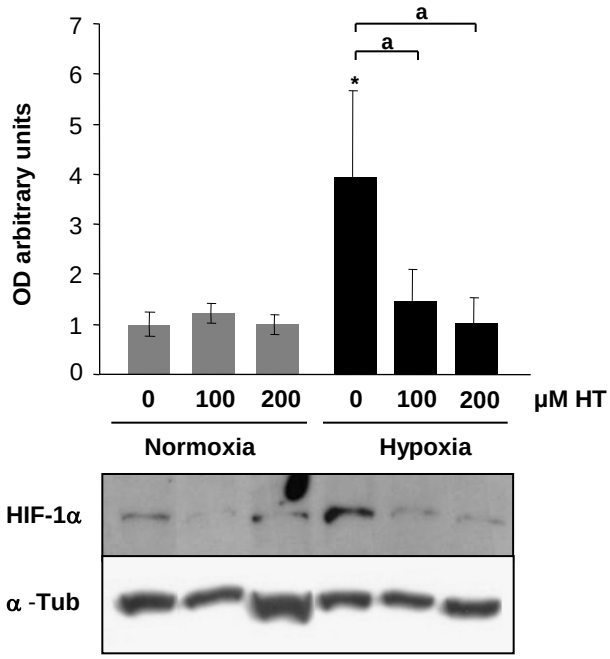

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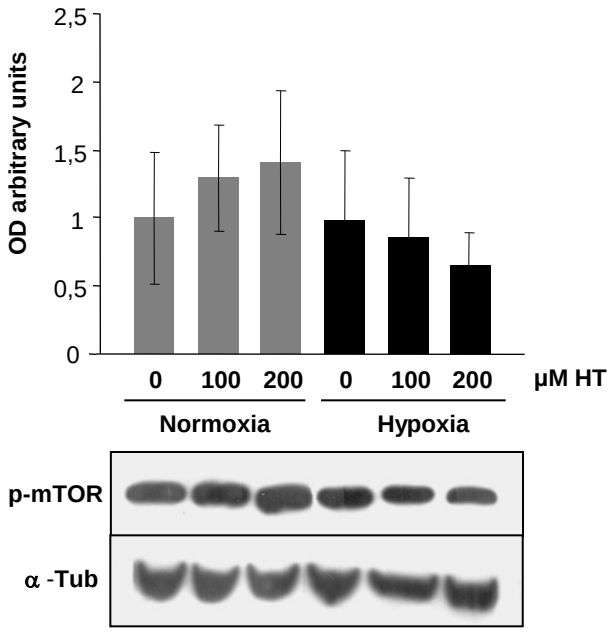

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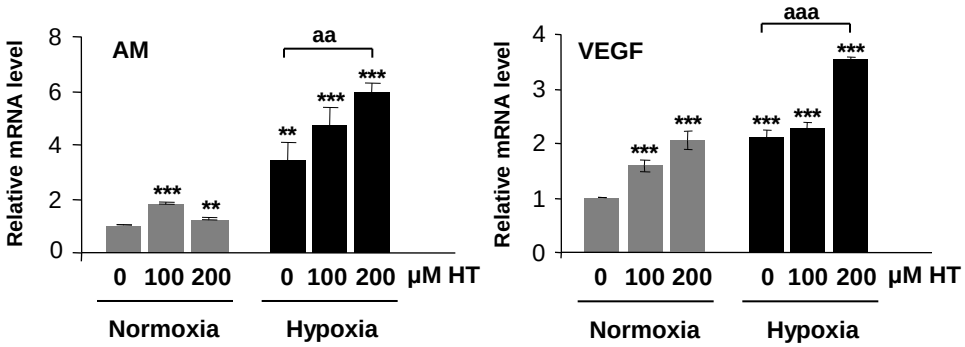

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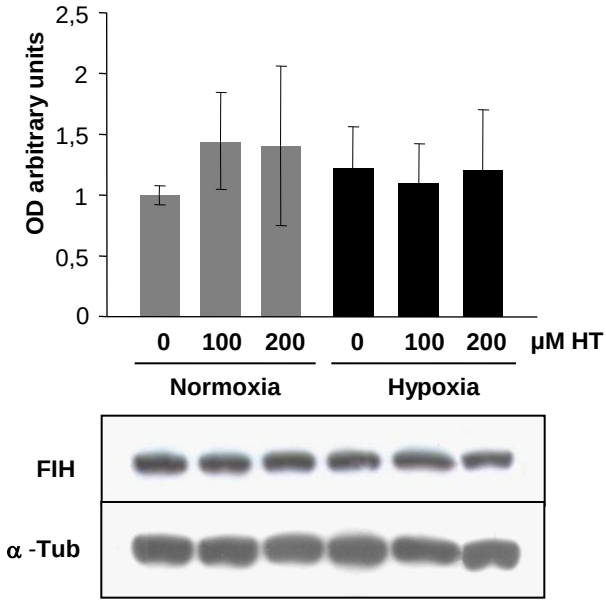

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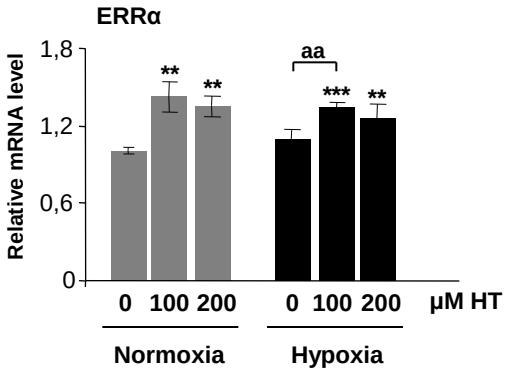
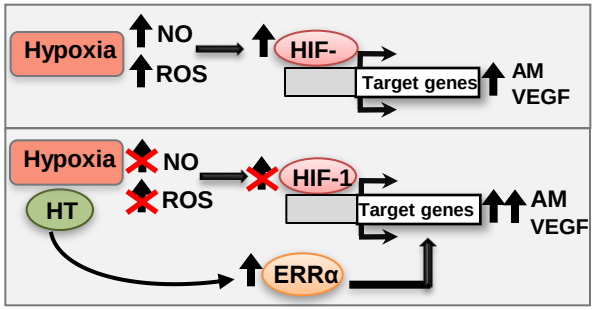


Figure 9



Graphical abstract

Proposed model of action of HT-treatment in the hypoxic response of sublethally damaged renal cells.