Author's response to reviews

Title: The cytoprotective drug amifostine modifies both expression and activity of the pro-angiogenic factor VEGF-A

Authors:

Sophie Dedieu (sophiededieu@gmail.com)
Xavier Canron (x.canron@hs.u-bordeaux1.fr)
Hamid Rezvani (hrRezvani@yahoo.com)
Marion Bouchecareilh (marionx2@hotmail.com)
Frederic Mazurier (frederic.mazurier@pmtg.u-bordeaux2.fr)
Roberta Sinisi (Roberta.Sinisi@polim.it)
Matteo Zanda (matteo.zanda@polimi.it)
Michel Moenner (m.moenner@angio.u-bordeaux1.fr)
Andreas Bikfalvi (a.bikfalvi@angio.u-bordeaux1.fr)
Sophie North (s.north@angio.u-bordeaux1.fr)

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Author's response to reviews: see over
Dear editor,

As requested, all the references to the word “hypoxia” concerning in vitro experiments performed at 3% oxygen concentration were replaced either by “low oxygen conditions”, “lowering oxygen concentrations” or “3% oxygen” both in the main text (p10 results section) and in the figures 1, 2 and S1 plus their corresponding legends. I hope that these modifications will allow the publication in BMC Medicine as soon as possible.

Sincerely,
Dr S. North

Changes in the main text p10, results section:

VEGF-A mRNA accumulation in response to WR-1065 is independent of HIF. HIF-1α and HIF-2α are involved in the transactivation of VEGF-A gene in response to hypoxia [42] and amifostine has been described as a potent hypoxia-mimetic compound that could activate HIF-1α both in vitro and in vivo [12]. Therefore, we analysed by Western Blot the accumulation of the hypoxia responsive transcription factors HIF-1α and HIF-2α in MCF7 cells treated with 1 mM WR-1065. As shown in figure 2A, WR-1065 treatment did not lead to the accumulation of either HIF-1α or HIF-2α in treated cells, whereas a strong increase in HIF-1α was observed in cells subjected to hypoxia. Consistently, 1 or 2 mM WR-1065 failed to induce any nuclear translocation of HIF-1α whereas hypoxia led to a significant increase of HIF-1α in the nucleus (Supplementary Figure S1).

We then measured the mRNA level of three representative HIF-1α-dependent genes, namely HK2, GLUT1. As expected, expression of these genes was strongly increased under hypoxia (Figure 2B). In comparison, their expression was significantly repressed (≈ 50%) in response to WR-1065 treatment, which suggest an HIF-1α-independent effect of WR-1065 on VEGF-A. In order to definitively exclude an HIF-1-dependent activation of VEGF-A by amifostine, we abolished HIF-1α protein expression by stably expressing a short hairpin RNA (shRNA) directed against the mRNA of HIF-1α [38]. MCF7 cells were then treated with WR-1065 or subjected to hypoxia. Under hypoxia, shRNA.HIF, but not shRNA.RFP, led to a strong inhibition of both HIF-1α protein (90%, Figure 2C) and VEGF-A mRNA accumulation (50%, Figure 2D). In response to WR1065 treatment, knock down of HIF-1α did not significantly modify the increase of VEGF-A mRNA expression. Altogether, these results indicate that the accumulation of VEGF-A mRNA in WR-1065-treated cells does not depend on HIF-1α activation.

Was replaced by:

VEGF-A mRNA accumulation in response to WR-1065 is independent of HIF. HIF-1α and HIF-2α are involved in the transactivation of VEGF-A gene in response to hypoxia [42] and amifostine has been described as a potent hypoxia-mimetic compound that could activate HIF-1α both in vitro and in vivo [12]. Therefore, we analysed by Western Blot the accumulation of the hypoxia responsive
transcription factors HIF-1α and HIF-2α in MCF7 cells treated with 1 mM WR-1065. As shown in figure 2A, WR-1065 treatment did not lead to the accumulation of either HIF-1α or HIF-2α in treated cells, whereas a strong increase in HIF-1α was observed in cells subjected to low oxygen concentration (3% instead of 20%). Consistently, 1 or 2 mM WR-1065 failed to induce any nuclear translocation of HIF-1α whereas lowering oxygen led to a significant increase of HIF-1α in the nucleus (Supplementary Figure S1).

We then measured the mRNA level of three representative HIF-1α-dependent genes, namely HK2, GLUT1. As expected, expression of these genes was strongly increased under low oxygen conditions (3% O₂ Figure 2B). In comparison, their expression was significantly repressed (≈ 50%) in response to WR-1065 treatment, which suggest an HIF-1α-independent effect of WR-1065 on VEGF-A. In order to definitively exclude an HIF-1-dependent activation of VEGF-A by amifostine, we abolished HIF-1α protein expression by stably expressing a short hairpin RNA (shRNA) directed against the mRNA of HIF-1α [38]. MCF7 cells were then treated with WR-1065 or subjected to low oxygen conditions (3%). Under low level of oxygen (3%), shRNA.HIF, but not shRNA.RFP, led to a strong inhibition of both HIF-1α protein (90%, Figure 2C) and VEGF-A mRNA accumulation (50%, Figure 2D). In response to WR1065 treatment, knock down of HIF-1α did not significantly modify the increase of VEGF-A mRNA expression. Altogether, these results indicate that the accumulation of VEGF-A mRNA in WR-1065-treated cells does not depend on HIF-1α activation.

Changes in the legend of Figure 1:

“Cells were then incubated in freshly added complete medium under normoxic conditions (NT, 21% O₂) or hypoxic conditions (H., 3% O₂), or in the presence of the indicated concentrations of WR-1065 under normoxia.”

Was replaced by:

“Cells were then incubated in freshly added complete medium under classical conditions of oxygen (NT, 20% O₂) or low oxygen conditions (LO, Low O₂, 3% O₂), or in the presence of the indicated concentrations of WR-1065 under 20% of oxygen.”

“VEGF-A protein secretion was measured by ELISA using supernatants of cells grown under hypoxia (3% O₂, □) or normoxia (○),”

was replaced by:

“VEGF-A protein secretion was measured by ELISA using supernatants of cells grown under low levels of oxygen (3% O₂, □) or 20% of oxygen (○),”

Changes in the legend of Figure 2.
“Cells were then incubated in complete medium with WR-1065 in the presence of aminoguanidine (AG) or were subjected to hypoxia (3% O₂ H)”.

Was replaced by:
“Cells were then incubated in complete medium with WR-1065 in the presence of aminoguanidine (AG) or were subjected to low levels of oxygen (3% O₂)”.

“Cells were treated for 6h under normoxia (20% O₂) either with or without 1 mM WR-1065; or under hypoxia (3% O₂).”

Was replaced by:
“Cells were treated for 6h under 20% of oxygen (20% O₂) either with or without 1 mM WR-1065; or low oxygen conditions (3% O₂).”

“Cells were either treated with 1 mM WR-1065 for 16h (upper panel, “+”), or exposed to hypoxia (H.) or normoxia (N.) for 6h (lower panel).”

Was replaced by:
“Cells were either treated with 1 mM WR-1065 for 16h (upper panel, “+”), or exposed to low oxygen conditions (3% O₂) or classical conditions (20% O₂) for 6h (lower panel).”

“Cells were incubated for 16h in the presence of increasing concentrations of WR-1065 or subjected to hypoxia (H.).”

Was replaced by:
“Cells were incubated for 16h in the presence of increasing concentrations of WR-1065 or subjected to low oxygen conditions (3% O₂).”

“Cells were treated with increasing doses of WR-1065, in the presence of AG, for 16h or exposed to hypoxia (H.) for the same period of time.”

Was replaced by:
“Cells were treated with increasing doses of WR-1065, in the presence of AG, for 16h or exposed to low oxygen conditions (3% O₂) for the same period of time.”

Changes in the legend of Figure S1
“MCF7 grown to 70% confluence on Lab-Tek chamber slides were treated for 6h with 4 mM Aminoguanidine alone (NT) or in combination with 1 mM Amifostine (WR-1065), or submitted to hypoxia: 3% O₂ for 6h (Hyp).”

Was replaced by:
“MCF7 grown to 70% confluence on Lab-Tek chamber slides were treated for 6h with 4 mM Aminoguanidine alone (O₂ 20%) or in combination with 1 mM Amifostine (WR-1065), or submitted to low oxygen conditions for 6h (O₂ 3%).”