Angiogenic Potential of 3-Nitro-4-Hydroxy Benzene Arsonic Acid (Roxarsone)

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BACKGROUND: Roxarsone (3-nitro-4-hydroxy benzene arsonic acid) is an arsenic compound widely used in the poultry industry as a feed additive to prevent coccidiosis, stimulate growth, and to improve tissue pigmentation. Little is known about the potential health effects from roxarsone released into the environment from chicken waste or from residual compound in chicken products.

OBJECTIVE: The growth potentiation and enhanced tissue pigmentation suggest that low levels of roxarsone exposure may have an angiogenic potential similar to that of inorganic arsenite (AsIII). The goal of this investigation was to test the hypothesis described above using cultured human aortic and lung microvascular endothelial cells in high-content imaging tube-forming assays and begin developing a molecular level understanding of the process.

METHODS: We used a three-dimensional Matrigel assay for probing angiogenesis in cultured human endothelial cells, and a polymerase chain reaction (PCR) array to probe the gene changes as a function of roxarsone or AsIII treatment. In addition, we used Western blot analysis for changes in protein concentration and activation.

RESULTS: Roxarsone was found to exhibit a higher angiogenic index than AsIII at lower concentrations. Increased endothelial nitric oxide synthase (eNOS) activity was observed for roxarsone but not for AsIII-induced angiogenesis. However, AsIII caused more rapid and pronounced phosphorylation of eNOS. Quantitative PCR array on select genes revealed that the two compounds have different and often opposite effects on angiogenic gene expression.

CONCLUSIONS: The results demonstrate that roxarsone and AsIII promote angiogenic phenotype in human endothelial cells through distinctly different signaling mechanisms.

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Concentration-responsive effects of roxarsone and As\textsuperscript{III} on the angiogenic potential of HAEC and HMVEC were compared in quantitative high-content cellular imaging tube-formation assays in Matrigel (Figure 1). Images of fluorescently labeled cells were analyzed by an algorithm that identified the angiogenic tubes formed by the association and clustering of the endothelial cells and quantitatively measured the properties of the identified tubes such as the number of tubes, tube lengths, tube areas, number of nodal branch points, and the angiogenic index—defined as the percentage of image area covered by tubes multiplied by 10 (Ghosh et al. 2007; Grove and Ghosh 2006). Each experiment was repeated 3 times, and each treatment was probed in at least six wells. Differences between treatments were analyzed by one- and two-way analysis of variance (ANOVA) and Dunnett or Bonferroni post hoc tests for significance using Graphpad Prism v4.0 software (Graphpad Software, San Diego, CA, USA).

**SuperArray Angiogenesis RT2 Profiler PCR array.** The effects of 24-hr exposures to As\textsuperscript{III} and roxarsone on HMVEC mRNA levels for 84 angiogenic and 5 constitutive genes were measured using the SuperArray quantitative polymerase chain reaction (PCR) assay (SuperArray Bioscience Corp., Frederick, MD, USA). Total RNA was extracted from approximately 500,000 cells using QIAGEN RNeasy (QIAGEN, Chatsworth, TN, USA) mini kits with inclusion of a DNase treatment step. First-strand cDNA synthesis and quantitative real-time PCR with SYBR green master mix was performed in an Opticon 2 DNA engine (BioRad Corp., Hercules, CA, USA) according to the manufacturer’s instructions.

**Western analysis.** Western blot analysis was performed on proteins isolated from HAEC exposed to As\textsuperscript{III} or roxarsone for 0.5, 1, or 4 hr. The proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene membranes, as previously described (Barchowsky et al. 1999b). Blots were probed with primary antibody to either total or ser1177 phosphorylated eNOS (Cell Signaling Technologies, Danvers, MA, USA) and reacted bands were detected by horseradish peroxidase–conjugated secondary antibodies and enhanced chemiluminescence substrates (PerkinElmer, Boston, MA, USA). Density of bands on film exposed to blots was determined using Scion Image software (Scion Corp., Frederick, MD, USA).

**Results and Discussion**

Concentration-responsive effects of roxarsone and As\textsuperscript{III} on the angiogenic potential of HAEC and HMVEC were compared in quantitative high-content cellular imaging tube-formation assays in Matrigel (Figure 1). Images of fluorescently labeled cells were analyzed by an algorithm that identified the angiogenic tubes formed by the association and clustering of the endothelial cells and quantitatively measured the properties of the identified tubes such as the number of tubes, tube lengths, tube areas, number of nodal branch points, and the angiogenic index. The angiogenic index is defined as the percentage of image area covered by tubes multiplied by 10 (Ghosh et al. 2007; Grove and Ghosh 2006). Both compounds were as effective as vascular endothelial cell growth factor (VEGF) in increasing the angiogenic index above the value for untreated control cells in both cell types (Figures 1 and 2). Both cell types exhibited strong angiogenic responses
with the arsenical treatments; however, the HMVEC showed a higher basal level of angiogenesis (Table 1). Because of their lower basal angiogenic activity, HAEC were used for additional mechanistic studies.

The angiogenic threshold for roxarsone was between 0.001 and 0.01 μM, an order of magnitude lower than the threshold for AsIII (Figures 1 and 2). As shown in in vivo chicken allantoic membrane angiogenesis assays (Soucy et al. 2003), above 1 μM AIII becomes toxic to tube formation and inhibits angiogenesis (Figure 2). In contrast, even 10 μM roxarsone showed no decrease in the angiogenic index (Figures 1 and 2) or signs of toxicity in the endothelial cells (data not shown). The fact that the efficacy for tube formation was similar, but the toxic potential differs, suggested that the two arsenicals differ in their mechanisms of action.

Nitric oxide (NO) production is required for certain stimuli to promote angiogenesis (Yu et al. 2005), but AsIII is known to promote reactive oxygen that limits available NO.

### Table 1. Angiogenic index of the response to arsenicals by different endothelial cells.

| Treatment                     | HAEC Mean ± SD | Negative control (%) | HMVEC Mean ± SD | Negative control (%) |
|-------------------------------|----------------|----------------------|-----------------|---------------------|
| No treatment (negative control) | 15 ± 3         | 100                  | 95 ± 62         | 100                 |
| Growth factor (positive control) | 35 ± 28       | 239                  | 125 ± 75        | 132                 |
| AsIII (0.1 μM)                | 32 ± 29        | 223                  | 146 ± 81        | 155                 |
| Roxarsone (0.1 μM)            | 28 ± 9         | 191                  | 198 ± 95        | 210                 |

* p < 0.05 and *** p < 0.001 determined from two-way ANOVA analysis, followed by the Bonferroni post-test.

Figure 3. Effects of L-NAME (A) and D-NAME (B) on angiogenic index. HAEC were treated as follows: (a) no treatment, (b) a mixture of growth factors as a positive control, (c) roxarsone (0.1 μM), and (d) AsIII (0.1 μM). Open bars represent cells with no NAME; blue bars represent cells treated with different isomers of NAME (50 μM). In each case, 12 wells were investigated, and each well was imaged at 5 different fields. Angiogenic index ± SD is plotted in each case.

* p < 0.05 and *** p < 0.001 determined from two-way ANOVA analysis, followed by the Bonferroni post-test.

Figure 4. Western blot showing phosphorylated eNOS (p-eNOS) (A), and total eNOS (B) in HAEC. Proteins were loaded equally after protein determination, which was confirmed by probing beta-actin (data not shown). Conditions: NC, no additional treatment; GF, a mixture of grown factors as positive control as described in Figure 2; Rox, 1 μM roxarsone; As, 1 μM AIII. All lanes on the gels were generated using proteins from separate cell cultures. Two replicates were done for each condition and were also used for statistical analyses. Individual bands were analyzed using Scion Image program, and the percent eNOS phosphorylated with respect to total is plotted for each condition (mean ± SD).

*p < 0.05 and *** p < 0.001 determined from two-way ANOVA analysis, followed by the Dunnett post-test.

Release in endothelial cells (Barchowsky et al. 1999a, Bunderson et al. 2006). The data in Figure 3 demonstrate that roxarsone requires NO generation to increase angiogenesis. Its effects were blocked by the eNOS inhibitor L-NG-nitroarginine methylster (L-NAME), but not its inactive enantiomer D-NG-nitroarginine methylster (D-NAME). In contrast, both NAME enantiomers prevented AIII from increasing the angiogenic index. This would suggest either that AIII inhibition by NAME was a nonstereospecific chemical effect or that D-NAME interacted with AIII in a unique manner relative to roxarsone. Thus, roxarsone and inorganic AIII appeared to have differential effects on signaling for NO-mediated angiogenesis.

To investigate a possible mechanism for increased eNOS activity after exposure to arsenicals, we exposed HAEC to either roxarsone or AsIII for 0.5–4 hr, then probed for levels of serine phosphorylated eNOS relative to total eNOS. As shown in Figure 4, both arsenicals caused time-dependent increases in Ser1177 phosphorylation of eNOS. However, roxarsone caused a progressive increase over 4 hr, whereas AsIII caused a rapid stimulation of phosphorylation that was declining by 4 hr. These data indicate that the arsenicals do not share similar common upstream signaling actions that enhance the activity of AKT, a serine/threonine protein kinase, which commonly accounts for serine phosphorylation (Dinnmeler et al. 1999; Fulton et al. 1999). In addition, the time course for the effects of AIII is consistent with its known ability to stimulate vascular NADPH oxidase to generate superoxide that quenches NO to form peroxynitrite (Bunderson et al. 2002; Lynn et al. 2000; Smith et al. 2001). These oxidants often mediate inflammatory angiogenesis (Ushio-Fukai and Alexander 2004) and thus may account for the mechanism of action for AIII.

Analysis of the effects of the two arsenicals on angiogenic gene activity further indicated that roxarsone and AIII signal through distinct but overlapping mechanisms. Supersaturation quantitative PCR arrays of total RNA extracts from HMVEC treated with either arsenical for 24 hr demonstrated that the arsenicals differentially affect expression of a limited set of 9 of 84 inducible genes measured. AIII induced more genes than it inhibited (Table 2). At 0.1 μM, roxarsone inhibited more genes than it induced (Table 2). Several genes were affected in the same direction, but not to the same degree by the two agents. Both arsenicals decreased angiogenic repressive interferon 1α (Strieter et al. 2005) and interferon-inducible CXCL9 transcripts. It was interesting that generally the higher roxarsone concentration (1.0 μM) had either no effect on gene expression or the opposite effect of the low concentration. The
exception was the concentration-dependent induction of hepatocyte growth factor (HGF) mRNA levels by both roxarsone concentrations. AsIII also strongly induced HGF tran-
scription. It is difficult to reconcile the differential inducer of angiogenesis than inorganic AsIII, demonstrate that roxarsone was a more potent inhibition to these differential effects, the fact that affected angiogenic gene induction. In addi-
tion, the analysis was limited by the small number of gene changes measured and a lack of informa-
tion regarding protein changes that may account for increased angiogenic index. Although beyond the scope of the current stud-
ies, additional mechanistic experiments would delineate the linkage between any gene or protein change and increased angiogenic index after arsenical exposure. Nonetheless, these data demonstrate that roxarsone and inorganic AsIII signal through different mechanisms to affect induction of genes that regulate angiogenesis.

**Conclusion**

Our results in human endothelial cell cultures demonstrate that roxarsone was a more potent inducer of angiogenesis than inorganic AsIII, and that roxarsone and AsIII signal through separate pathways to promote angiogenesis. The arsenicals had different time courses for disease risk. Future studies are needed to account for increased angiogenic index.

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sis-blocked tetraploid cells using high content analysis. Assay Drug Dev Technol 4:421–442.

**Table 2. SuperArray Angiogenesis RT² Profiler PCR array for angiogenic genes.**

| Genes                | Fold control |
|----------------------|--------------|
|                      | 0.1 µM roxarsone | 1.0 µM roxarsone | 1.0 µM AsIII |
| **Proangiogenic genes** |
| Angiopoietin-1       | 0.46 ± 0.25*   | 0.77 ± 0.25      | 0.67 ± 0.14  |
| CXCL3                | 0.95 ± 0.25    | 1.38 ± 0.52      | 1.74 ± 0.10* |
| Hepatocyte growth factor | 1.99 ± 0.63*  | 2.70 ± 0.38**    | 4.20 ± 0.39*** |
| Insulin-like growth factor 1 | 0.84 ± 0.54    | 0.90 ± 0.37      | 2.47 ± 0.63** |
| Leukocyte cell-derived chemokatin 1 | 0.46 ± 0.23*   | 0.96 ± 0.20      | 0.55 ± 0.09* |
| Leptin               | 0.21 ± 0.08**  | 0.98 ± 0.43      | 0.57 ± 0.29  |
| Plasminogen          | 0.31 ± 0.02**  | 0.94 ± 0.18      | 0.63 ± 0.30  |
| **Anti-angiogenic genes** |
| Interferon 1α        | 0.34 ± 0.09*   | 1.14 ± 0.39      | 0.71 ± 0.32  |
| CXCL9                | 0.17 ± 0.08**  | 1.03 ± 0.33      | 0.46 ± 0.13** |

Approximately 500,000 HMVEC were incubated with roxarsone or AsIII for 24 hr. Extracted total RNA was then isolated and mRNA levels for 84 inducible and 5 constitutive genes were measured, according to the manufacturer’s instructions. Data are presented as mean ± SD fold over untreated control for PCR products normalized to housekeeping gene expression.

*p < 0.05, **p < 0.01, and ***p < 0.001 (n = 6 separate RNA extracts).