

## Diverse Contributions From the Initial Discovery of Mechanisms of Angiotensin II–Induced Oxidation in Smooth Muscle Cells

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### Angiotensin II Stimulates NADH and NADPH Oxidase Activity in Cultured Vascular Smooth Muscle Cells

Griendling et al  
*Circ Res.* 1994;74:1141–1148.

**This article reported that effects of angiotensin II on hypertrophy in vascular smooth muscle cells were attributable to activation of NADPH oxidases. This finding has been an initial stimulant to increasing understanding that angiotensin II–induced oxidative process contributes to many characteristics of vascular smooth muscle cell responses.**

The article “Angiotensin II Stimulates NADH and NADPH Oxidase Activity in Cultured Vascular Smooth Muscle Cells” by Griendling et al<sup>1</sup> has the distinction of being in the top echelon of highly cited publications in *Circulation Research*. In addition to the high number of total citations (1762 as of October 2013), equally impressive is its continuous impact on contemporary literature since its publication. To place this number of citations in context, among the 22 145 articles published in 1994, which were categorized under Cardiovascular System and Cardiology in the ISI Web of Science, only 4 have been cited >1000 times. The Griendling et al article is ranked the top and has been cited 35% more frequently than the second most cited article. Moreover, compared with the 264 articles published in 1994 in *Circulation Research*, the citation frequency of Griendling et al article is far greater than the mean (Figure 1). The consistently high citation rate has been attributed to the enduring relevance of intracellular signaling mechanisms of vascular smooth muscle cell (VSMC) biology and also that the article provided valuable information on angiotensin II (AngII)-induced signaling pathways. It is worth noting that this article has had an impact on diverse research fields, in addition to its significant influence on cardiovascular research. In addition, the repercussion of this article was further extended by the many research articles, reviews, and editorials that cited this work of Griendling et al.

AngII has multiple effects on VSMCs, which include stimulating contraction, proliferation, hypertrophy, and extracellular matrix production.<sup>2</sup> The wide spectrum of AngII-induced effects has led to a voluminous literature dealing with

intracellular signaling pathways and their relative importance.<sup>2</sup> Griendling and colleagues have an impressive track record for their contributions to this literature. Griendling and colleagues’ earlier work reported AngII-induced activation of phospholipase D in VSMCs with subsequent generation of phosphatidic acid and diacylglycerol.<sup>3</sup> Additionally, studies in neutrophils demonstrated that phosphatidic acid activated NADPH oxidase to result in rapid generation of huge amounts of superoxide ions.<sup>4</sup> At the time of these publications, there was evolving evidence that specific reactive oxidant species could act as mediators of intracellular signaling.<sup>5</sup> Taken together, this led to a rationale for determining the effects of AngII stimulation in VSMCs on reactive oxygen species–generating systems.

Studies by Griendling et al<sup>1</sup> were performed in cultured VSMCs from rat thoracic aortas using a multifaceted approach with several analytic methods and enzyme modulators. Incubation of VSMCs with AngII led to AngII type 1 receptor–mediated augmentation of superoxide formation that increased gradually for a 6-hour interval. By comparing the effects of cell permeable versus impermeable inhibitors, it was concluded that AngII-increased superoxide production originated from intracellular sources. This gradual increase in intracellular superoxide production contrasts the rapid extracellular release in activated neutrophils.<sup>4</sup> Preliminary studies with oxidase inhibitors led to a focus on NADH and NADPH oxidases as the source of superoxide. In agreement with this focus, there was a similarity between AngII-induced intracellular superoxide production rates and simulation rates and peak response intervals of these 2 oxidases. Furthermore, subcellular localization in combination with activators and inhibitors of NADH and NADPH oxidases demonstrated that AngII-induced superoxide was generated at the plasma membrane through activation of NADH and NADPH oxidases. This pathway was related to AngII stimulation in VSMCs, as demonstrated by NADH and NADPH oxidase inhibitors profoundly decreasing AngII-induced VSMC hypertrophy. This initial publication attributed the predominance of superoxide formation to NADH oxidase in VSMCs, rather than to NADPH oxidase. However, in subsequent studies to refine the protocol for superoxide measurement using lucigenin, the authors demonstrated that, contrary to the conclusion of their initial report, NADPH oxidase was the major source of superoxide in VSMCs.<sup>6</sup> The initial publication and the authors’ subsequent elaborations have provided major insights into the pathway of AngII-induced superoxide production in VSMCs.

The basis for the high and enduring citation rate for this article<sup>1</sup> is that it laid foundation to several fields that have flourished in the past 2 decades. First, superoxide measurement has been an area of controversy. This article<sup>1</sup> provides a detailed protocol of the lucigenin-based assay. More importantly, extensive studies were performed to verify the authenticity of

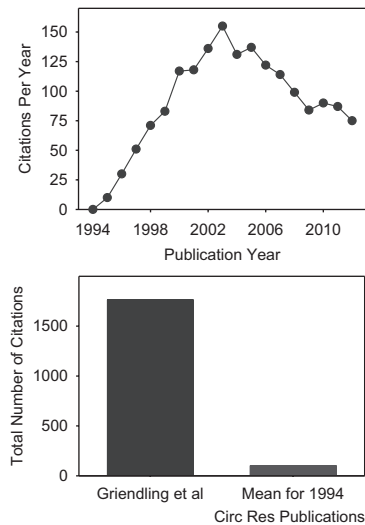
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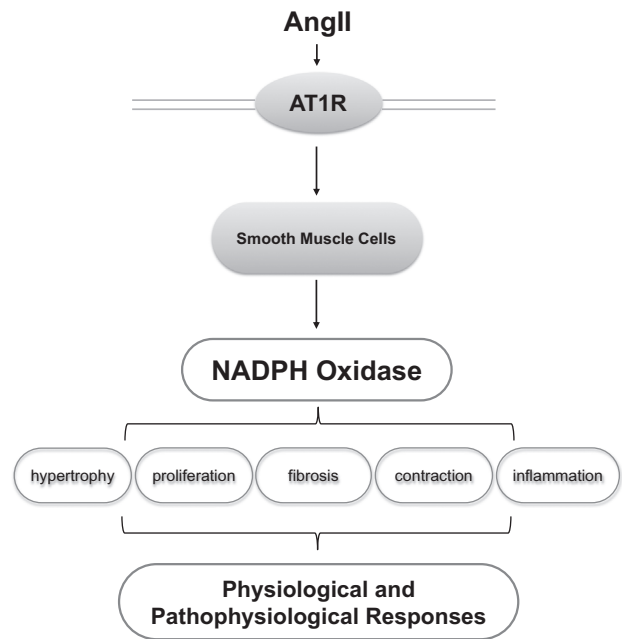


**Figure 1. Summary of the impact of the article by Griendling et al.<sup>1</sup> A, Annual citation rate. B, Total number of citations for Griendling et al compared with the mean number of citations for all the 264 articles published in *Circulation Research* in the same year. (Source: Thomson Reuters Web of Science; updated in October 2013).**

this lucigenin measurement of superoxide.<sup>1</sup> Therefore, investigators who attempt to replicate this assay frequently cite this article.<sup>1</sup>

Second, this article<sup>1</sup> provides a base to understand the importance of the NADPH oxidase complex. Although it was originally thought that NADPH oxidase was a multimeric complex, it was subsequently found that there are variants of specific proteins and different forms of the complex. The originally identified NADPH oxidase complex consisted of 2 membrane intercalated proteins, p22phox and gp91phox, coupled with other cytoplasmic proteins, p47phox, p67phox, and Rac. However, it is now recognized that there are multiple isoforms of gp91phox, namely, Noxs and Duoxs.<sup>7,8</sup> The major functional isoform in VSMCs is Nox1, which is associated with p22phox. Nox4 is also present in VSMCs and associated with p22phox. Therefore, investigators who study the regulation and consequences of Nox proteins frequently cite this article.<sup>1</sup>

Third, this article<sup>1</sup> propelled research in several aspects of VSMCs, particularly those related to intracellular signaling mechanisms. This encompasses definitions of both upstream and downstream components of AngII stimulation on NADPH oxidase activity in VSMCs.<sup>2,8</sup> In addition to superoxide anion production, AngII promotes NADPH oxidase-derived hydrogen peroxide production in VSMCs.<sup>9–11</sup> Pathways that are being invoked include protein kinase C, p38 mitogen-activated protein kinase, and protein kinase B/Akt. Furthermore, the importance of NADPH oxidases in determining AngII effects has also been recognized in other cell types, including endothelial cells, fibroblasts, and inflammatory cells.<sup>2,9,10,12,13</sup> Since the publication of this article,<sup>1</sup> effects of AngII induction on multiple cell types have been investigated, and new signaling mechanisms related to this signaling pathway leading to the production of reactive oxygen species have been discovered (Figure 2).<sup>2</sup>



**Figure 2. Summary of the diversity of AngII-induced physiological and pathophysiological responses attributable to NADPH activation in vascular smooth muscle cells, subsequent to the study of Griendling et al.<sup>1</sup>**

Fourth, the impressive citation rate of this article<sup>1</sup> could also be attributed to the increasing interest in the manipulation of oxidant processes for potential therapeutic benefits. There is compelling evidence that AngII-augmented oxidation contributes to cardiovascular pathologies, including hypertension,<sup>12,14</sup> vascular hyperplasia and hypertrophy,<sup>11,14</sup> and aortic aneurysms.<sup>15</sup> In addition, this article has provided significant understanding on the complex mechanisms by which pharmacological inhibition of the renin–angiotensin system has profound effects on multiple cardiovascular and other diseases.<sup>2,16</sup> Overall, *Circulation Research* has been fortunate to have published this article by Griendling et al.<sup>1</sup> The mechanistic insights into cardiovascular research and many other research areas provided by this article are the basis for its enduring relevance and its consistently high citation rate.

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