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Role of macrophage PPARγ in experimental hypertension

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Kriska T, Cepura C, Gauthier KM, Campbell WB. Role of macrophage PPARγ in experimental hypertension. Am J Physiol Heart Circ Physiol 306: H26–H32, 2014. First published October 25, 2013; doi:10.1152/ajpheart.00287.2013.—Targeted disruption of the Alox15 gene makes mice resistant to angiotensin II-, DOCA/salt-, and Nω-nitro-l-arginine methyl ester (l-NAME)-induced experimental hypertension. Macrophages, a primary source of Alox15, are facilitating this resistance, but the underlying mechanism is not known. Because Alox15 metabolites are peroxisome proliferator-activated receptor (PPAR)γ agonists, we hypothesized that activation of macrophage PPARγ is the key step in Alox15 mediation of hypertension. Thioglycollate, used for macrophage elicitation, selectively upregulated PPARγ and its target gene CD36 in peritoneal macrophages of both wild-type (WT) and Alox15−/− mice. Moreover, thioglycollate-injected Alox15−/− mice became hypertensive upon l-NAME treatment. A similar hypertensive effect was observed with adoptive transfer of thioglycollate-elicited Alox15−/− macrophages into Alox15−/− recipient mice. The role of PPARγ was further specified by using the selective PPARγ antagonist GW9662. WT mice treated with 50 μg/kg daily dose of GW9662 for 12 days became resistant to l-NAME-induced hypertension. The PPARγ antagonist treatment also prevented l-NAME-induced hypertension in thioglycollate-elicited Alox15−/− mice, indicating a PPARγ-mediated effect in macrophage elicitation and the resultant hypertension. These results indicate a regulatory role for macrophage-localized PPARγ in l-NAME-induced experimental hypertension.

The immune system has been implicated in the pathogenesis of hypertension for decades, but there is currently no definitive answer as to whether hypertension is an immunologic disease. Growing evidence suggests that components of both the adaptive and innate immune systems are involved (9). The presence of T lymphocytes is necessary for the development of angiotensin II and DOCA-salt induced hypertension (8, 26). The role of T cells is further supported by demonstration that immunosuppressive treatment reduces blood pressure in various hypertensive rat models (18, 23). Monocytes/macrophages, on the other hand, are activated by vasoactive factors such as angiotensin II, endothelin-1, and nitric oxide, leading to target organ infiltration and damage (10).

Genetic alteration of macrophages also leads to resistance against hypertension in mice (13). We recently demonstrated that mice lacking macrophage-localized Alox15 [previously known as 12/15-lipoxygenase (LO)] are resistant to several types of experimental hypertension (15). Macrophage Alox15 plays both a pro- and anti-inflammatory role by oxidizing LDL or generating lipid mediators involved in the resolution of inflammation (30). Among bioactive compounds, the oxidized metabolites of arachidonic acid [15-hydroxyeicosatetraenoic acid (15-HETE)] and linoleic acid [9-hydroxyoctadecadienoic acid (9-HODE); 13-HODE] are produced most abundantly (11, 20).

Macrophages are involved in many aspects of the adaptive and innate immune systems that are coordinated by nuclear hormone receptors. These nuclear receptors regulate transcription, shape the immune response, and are activated by lipid signaling (19). Because HETEs and HODEs are potent activators of peroxisome proliferator-activated receptor (PPAR)γ and PPARγ knockout mice exhibit hypotension (6), we hypothesized that the pathway leading to the development of experimental hypertension requires an activation of this nuclear receptor.

In the current study, we used the Alox15−/− mouse model to investigate the role of macrophage PPARγ in Nω-nitro-l-arginine methyl ester (l-NAME)-induced hypertension. Macrophage elicitation was chosen as an important experimental tool after discovering that thioglycollate treatment of Alox15−/− mice upregulated macrophage PPARγ and its target gene CD36 and resulted in hypertension with l-NAME treatment. These effects were abolished by using selective PPARγ antagonist GW9662. The PPARγ antagonist-treated wild-type (WT) mice also became resistant against l-NAME-induced hypertension, indicating that macrophage-localized PPARγ is an important checkpoint in the development of l-NAME-induced hypertension.

MATERIALS AND METHODS

Animals. C57BL6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME), and Alox15−/− mice on a C57BL6 background were provided by Dr. J. L. Nadler of East Virginia Medical School. All animals were genotyped using PCR with the following primers: common forward 5′-GGG TCG CTG CTG AGG TAC AG-3′, knockout reverse, 5′-GGG AGG ATT GGG AAG ACA AT-3′, and WT reverse 5′-CCA TAG AGG AGA CCA GCA CA -3′ (14). Products from WT and Alox15−/− animals were 417 and 200 bp, respectively. Eight- to ten-week-old males (20–25 g) were used. Animal protocols were approved by the Animal Use and Care Committee of the Medical College of Wisconsin. Procedures were performed in accordance with the National Institutes of Health Guide for the Use of Laboratory Animals.

Blood pressure measurement. Systolic blood pressure of C57BL6 (WT) and Alox15−/− control or l-NAME-induced hypertensive mice were determined using tail-cuff method with IITC Life Sciences Blood Pressure System (Woodland Hills, CA) (14). Mice were acclimatized to the blood pressure chamber twice a week starting from 6 wk of age. Recordings were started after allowing the animals to acclimate in chambers for 10–15 min. Three successful measurements were averaged as a single data point in each session. The reported
systolic blood pressure represents an average of data collected during 3 consecutive days of measurement.

**Experimental hypertension model.** In the l-NNAME-induced hypertension model, mice received 100 mg·kg⁻¹·day⁻¹ of G-nitro-L-arginine-methyl ester in regular drinking water for 6 days (14). The water intake was monitored during this time period to exclude any possible differences between experimental groups. The 7-day cumulative water intake values were 29.1 ± 2.1 ml and 26.6 ± 3.1 ml for WT and Alox15⁻/⁻ groups, respectively. The total doses of l-NNAME consumed over the 7 days were 29.1 ± 2.1 mg and 26.6 ± 3.1 mg for WT and Alox15⁻/⁻ groups, respectively. Systolic blood pressure was monitored with the tail-cuff method (see above). Individual differences in pre- and post-l-NNAME values of systolic blood pressure of each animal were averaged within an experimental group.

**Tissue preparation.** Aortas and kidneys were dissected and cleansed of connective tissue in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer of (in mM) 10 HEPES, 150 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, and 6 glucose (pH 7.4). Elicited macrophages were harvested from euthanized mice by peritoneal lavage using sterile PBS of (in mM) 100 NaCl, 3 KCl, 10 Na₂HPO₄, and 2 KH₂PO₄ (pH 7.4). To obtain elicited macrophages mice were injected intraperitoneally with 2 ml of sterile 3.85% Thiglycollate Medium Bremer Modified medium (Becton Dickinson) 3 days before isolation. Non-elicited macrophages were harvested by peritoneal lavage of untreated, euthanized mice. All tissues were used immediately or snap-frozen in liquid nitrogen immediately after dissection and stored at −80°C until further use.

**Western immunoblotting.** Tissues frozen in liquid nitrogen were pulverized and homogenized in lysis buffer containing (in mM) 10 HEPES, 150 NaCl, 1 EDTA, 1 EGTA, and 1 sodium bisulfite (pH 7.5) containing Complete Mini cocktail protease inhibitor (Roche Diagnostics) and 0.5% Triton X-100. Homogenates were further incubated on ice for 30 min with occasional vortexing. Macrophage pellets were resuspended and homogenized directly in lysis buffer. The homogenates were centrifuged at 12,000 g for 20 min, and the supernatants were used for analysis. Then, 50 μg of total protein were applied to each lane of a 10% SDS-PAGE gel (Criterion Precast Gel; Bio-Rad), separated, and transblotted onto nitrocellulose membrane. After blocking with 5% nonfat dry milk (Bio-Rad) in TBS-T buffer (20 mM Tris base, 150 mM NaCl, and 0.1% Tween-20), membranes were probed with rabbit polyclonal anti-12-LO primary antibody generated in our laboratory (7, 28), with mouse monoclonal anti-PPARγ primary antibody (Santa Cruz Biotechnology), or with rabbit polyclonal anti-CD36 antibody (Novus Biologicals) all in 1:1,000 dilution. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit (GE Healthcare) or goat anti-mouse IgG (Invitrogen) was used as secondary antibody, both in 1:10,000 dilution. β-Actin was used as a loading control detected by mouse monoclonal anti-β-actin primary antibody (1:5,000 dilution; Santa Cruz Biotechnology) and an HRP-conjugated goat anti-mouse secondary antibody (1:10,000 dilution; Invitrogen). Immunoreactive bands were visualized using SuperSignal West Pico or West Femto chemiluminescence reagents (Thermo Scientific).

**Adoptive macrophage transfer.** Elicited peritoneal macrophages were harvested from donor Alox15⁻/⁻ mice injected with sterile thioglycollate solution 3 days earlier. Cells were washed and resuspended in PBS to ~8 million cells/ml. Recipient Alox15⁻/⁻ mice were injected intraperitoneally with either 1 ml of PBS or 1 ml of the macrophage suspension. Immediately after injection mice were treated for 6 days with l-NNAME to induce hypertension (see above).

**Flow cytometry.** The distribution of cell types in the elicited or nonelicited peritoneal lavage was determined by flow cytometry using monoclonal antibodies (Biolegend) to monocytes/macrophages (anti-CD11b-APC-Cy7 and anti-F4/80-PE), T cells (anti-CD3-PE-Cy7), and B cells (anti-B220-APC). The cells were stained simultaneously with each antibody for 20 min on ice. They were then washed twice in PBS containing 0.5% BSA (fluorescence-activated cell sorting (FACS) buffer) and resuspended in FACS buffer for analysis. Fluorescence minus one (FMO) controls were used to ensure an accurate measurement of surface marker expression. Cell suspensions were analyzed using a BD FACS Aria SRS instrument in the Medical College of Wisconsin Flow Cytometry Core Facility. The data were analyzed using CellQuest Pro software.

For the 12-LO immunoblot analysis the stained cell suspensions were gated and sorted collecting the macrophage, T cell, and B cell populations. Between 0.5 and 1.0 million cells from each type were used for further analysis.

**Statistics.** Data are presented as means ± SE. The two-tailed Student’s t-test or ANOVA was used for determining the significance of observed differences between experimental values, with P < 0.05 considered statistically significant.

**RESULTS**

Expression of Alox15 protein in cells of the peritoneal lavage. Peritoneal lavage from control (nonelicited) or thioglycollate-injected (elicited) mice were analyzed for the distribution of macrophages and T or B lymphocytes using flow cytometry. As shown on Fig. 1A, thioglycollate elicitation shifted the macrophage-to-lymphocyte ratio from 55%-to-45% to a 88%-to-12%. The actual cell numbers in each cell type category indicated that the observed shift in cell composition was caused by an increase in the number of macrophages. The number of T or B cells did not change with thioglycollate-elicitation (data not shown). Sorted cells from the elicited lavage were analyzed for Alox15 protein expression. Macrophages were the only cell type that exhibited Alox15 expression. In both T and B lymphocytes, the Alox15 signal was undetectable (Fig. 1B). There was no difference in the levels of Alox15 protein expression between elicited and nonelicited macrophages (data not shown). These results indicate that the
disruption of the Alox15 gene affected only the macrophage population, making any direct contribution of T or B lymphocytes unlikely.

Effect of thioglycollate elicitation on Alox15+−/− macrophages. Systolic blood pressure was measured in male C57BL6 WT and Alox15+−/− mice using the tail-cuff method. There was no significant difference in basal blood pressure between the two groups. Systolic blood pressures ranged between 94 and 98 mmHg (Fig. 2, gray bars) indicating that Alox15 gene disruption had no effect on basal blood pressure. When hypertension was induced by L-NAME treatment, the blood pressure of WT animals significantly increased to 140 mmHg between the fifth and seventh day of L-NAME ingestion; however, the blood pressure of Alox15+−/− mice remained unchanged (Fig. 2, black bars). This resistance to L-NAME-induced hypertension was abolished by adoptive transfer of elicited Alox15+−/− macrophages into Alox15+−/− hosts. The adoptive transfer of elicited macrophages caused the blood pressure to rise to 122 mmHg (Fig. 2). These data indicate that elicitation induced changes in Alox15 knockout macrophages that resulted in elevation in the blood pressure of Alox15+−/− mice upon a hypertensive stimulus.

PPARγ and CD36 protein expression in elicited versus nonelicited macrophages and T cells. Nonelicited WT or Alox15+−/− peritoneal macrophages have low expression of PPARγ protein. Upon thioglycollate elicitation both WT and Alox15+−/− macrophages exhibited a robust upregulation in PPARγ expression detected by Western immunoblot (Fig. 3, first 2 columns of top immunoblot panels). The same pattern was detected for the PPARγ-regulated gene CD36. Thioglycollate elicitation dramatically upregulated the expression of CD36 protein compared with nonelicited macrophages (Fig. 3, first 2 columns of bottom immunoblot panels). In T cells, on the other hand, thioglycollate elicitation failed to induce any measurable PPARγ or CD36 expression (Fig. 3, third column of immunoblot panels). These results indicate that in macrophages, thioglycollate elicitation upregulates PPARγ and PPARγ-regulated genes independent of the presence or absence of the Alox15 enzyme. Unlike macrophages, T cells were not affected by thioglycollate elicitation, making them unlikely candidates to facilitate hypertension.

Role of PPARγ in the development of L-NAME-induced hypertension. Because the thioglycollate-induced upregulation of PPARγ coincided with the acquired sensitivity of Alox15+−/− mice toward L-NAME-induced hypertension, we determined the effect of PPARγ inhibition on L-NAME hypertension. Systolic blood pressure was monitored in WT and Alox15+−/− mice that were injected daily with an irreversible PPARγ antagonist, GW9662, or vehicle for 12 days. The GW9662 did not cause any change in blood pressure compared with pre-injection baseline or vehicle in either WT or Alox15+−/− mice (Fig. 4, gray bars). At the end of the injection period, mice were treated with L-NAME for 7 days. In the vehicle-treated WT animals, L-NAME caused a significant elevation in blood pressure. GW9662 treatment abolished the blood pressure elevation, and blood pressure remained at the baseline level (Fig. 4A, black bars). The effect of the PPARγ antagonist on WT peritoneal macrophages was evaluated by

Fig. 2. Effect of thioglycollate-elicited Alox15+−/− macrophages on Nω-nitro-l-arginine methyl ester (L-NAME)-induced hypertension in Alox15+−/− mice. Hypertension was induced with L-NAME treatment in wild-type (WT) or Alox15+−/− mice and in Alox15+−/− recipient mice that were injected with 8 × 10^6 thioglycollate-elicited Alox15+−/− macrophages. Blood pressure was monitored by the tail-cuff method. Each value represents mean ± SE; n = 4 for PM- or vehicle-injected Alox15+−/− mice and n = 8 for all other groups. *P < 0.01 compared with control.

Fig. 3. Effect of thioglycollate elicitation on peroxisome proliferator-activated receptor (PPAR)γ and CD36 protein expression in peritoneal macrophages and T cells. Macrophages and T cells from control (−TG) or thioglycollate-injected (+TG) WT and Alox15+−/− mice were harvested and subjected to Western immunoblot analysis. β-Actin was used as a loading control. Representative blots of 3 experiments for Alox15+−/− and 4 experiments for WT cells are shown. Relative densitometric values for each Western immunoblot of PPARγ (left) or CD36 (right) are shown. *P < 0.01 compared with nonelicited control; **P < 0.001 compared with nonelicited control.
Western immunoblot. After GW9662 injections, mice were treated with thioglycollate. GW9662 treatment significantly reduced the thioglycollate induction of PPARγ (Fig. 4B).

Alox15−/− mice were resistant to l-NAME-induced hypertension as previously shown (Fig. 2). Consequently, the blood pressure of vehicle-injected Alox15−/− mice stayed at baseline level, as did the GW9662-injected mice (Fig. 4C, black bars). Thioglycollate treatment of vehicle-treated Alox15−/− mice resulted in a significant blood pressure increase, which was abolished by PPARγ antagonist in a manner similar to that observed in WT mice (Fig. 4D, black bars). These results suggest that active macrophage PPARγ is required for l-NAME-induced hypertension to develop.

Macrophage specificity of the upregulation of PPARγ in thioglycollate-injected mice. To assess the specificity of effect of thioglycollate injection, we compared PPARγ expression in mouse organs that play key role in hypertension. Aortas, peritoneal macrophages, and kidneys of control or thioglycollate-injected animals were analyzed by Western blot. The PPARγ protein expression of aortas and kidneys of thioglycollate-injected mice did not change significantly compared with control, untreated mice (Fig. 5, left). Peritoneal macrophages, on the other hand, exhibited a robust upregulation of PPARγ expression. Thioglycollate injection caused an even more dramatic upregulation of CD36 protein in macrophages, while leaving the expression levels unchanged in aorta and kidney (Fig. 5, right). These results indicate that intraperitoneal thioglycollate treatment causes macrophage-specific PPARγ upregulation in mice.

**DISCUSSION**

The Alox15 enzyme oxygenates polyunsaturated fatty acids and phospholipids of biological membranes (16). It is expressed in macrophages and plays a crucial role in macrophage functions that are related mostly to atherosclerosis (12) and inflammatory responses (5). To study these processes, a global Alox15 knockout mouse model was developed (12, 27). Recently, the Alox15−/− mice were found to be resistant to several types of experimental hypertension (1, 15), leading to the hypothesis that macrophages represent a regulatory checkpoint in the pathway to hypertension. To prove this hypothesis, we demonstrated that macrophage depletion with clodronate results in resistance against l-NAME-induced hypertension in mice (15). The susceptibility to l-NAME-induced hypertension in Alox15−/− mice was restored by adoptive transfer of WT or thioglycollate-activated Alox15−/− peritoneal macrophages. These findings emphasize the central role for macrophages in experimental hypertension.

These studies should not be interpreted to imply that macrophage nitric oxide synthase or macrophage-derived nitric oxide contribute to l-NAME-induced hypertension. In the l-NAME-induced hypertension model, inhibition of endothelial nitric oxide synthase results in endothelial dysfunction and hypertension (14, 21). We considered the possibility that alterations in macrophage nitric oxide and/or reactive oxygen species production contribute to the resistance of Alox15−/− mice to l-NAME hypertension. Therefore, we compared the superoxide production of WT and Alox15−/− macrophages...
and found no difference. Nitric oxide production by WT and Alox15−/− macrophages was measured by chemiluminescence after LPS induction. Both WT macrophages produced 4 nmol nitrite/mg protein, and the production by Alox15−/− macrophages was ~15% higher than WT macrophages (data not shown). Thus neither superoxide nor nitric oxide production by macrophages can explain the resistance to hypertension by Alox15−/− mice.

T lymphocyte deficiency also results in resistance against experimental hypertension (8, 26). However, Alox15 is not expressed in lymphocytes so must not be involved in the T cell contribution to hypertension. Involvement of T cells could not be ruled out in our studies since T cells and macrophage cross talk occurs. One such cross-talk point that involves Alox15 is the production of cytokine IL-4 by Th2-activated T cells, since macrophage-derived IL-6 induces a Th1 to Th2 switch (4). Experimental data suggest that the IL-6 production is reduced in macrophages from Alox15−/− mice (29). Furthermore, IL-6−/− mice are resistant to angiotensin II-induced hypertension (2, 17) indicating a direct role for IL-6 in mediating the hypertensive responses.

Alox15 has a physiological role in the generation of endogenous ligands for PPARγ in macrophages. Inhibition of the enzyme or destruction of the Alox15 gene downregulates the PPARγ-regulated genes such the CD36 scavenger receptor (11) (Fig. 5). Based on this finding, we hypothesized that the development of experimental hypertension requires a functional Alox15-PPARγ pathway, and the lack of components of this pathway results in the resistance to hypertension. To test this hypothesis, a series of in vivo experiments were conducted using GW9662, an irreversible PPARγ inhibitor. Pharmacological blockade of PPARγ in L-NAME-treated WT mice had the same effect on systolic blood pressure as knockout of the Alox15 gene, i.e., GW9662 prevented any blood pressure elevation with L-NAME treatment.

Thioglycollate-induced macrophage elicitation was originally used to boost the macrophage production; however,
elicitation evolved into a useful tool in studying the role of PPARγ in experimental hypertension. Upon thioglycollate treatment macrophages become activated, characterized by enhanced migration and secretory activity, but have impaired microbicidal or tumoricidal activity (3). We also noticed that thioglycollate dramatically upregulated both PPARγ and CD36 protein expression in macrophages from either WT or Alox15−/− mice. This upregulation was accompanied by regained susceptibility of Alox15−/− mice towards l-NAME-induced hypertension (Fig. 6). The possibility that thioglycollate allows the development of hypertension by a PPARγ-independent pathway was ruled out by the adoptive transfer experiment. Alox15−/− recipient mice became hypertensive upon receiving thioglycollate-elicted macrophages harvested from Alox15−/− donors. Pretreatment of Alox15−/− mice with the PPARγ inhibitor prevented any thioglycollate-facilitated blood pressure elevation in a similar fashion as in WT animals. In a preliminary study, the intraperitoneal injection of 15-HETE, a major Alox15 metabolite and PPARγ agonist, conferred susceptibility to l-NAME hypertension in Alox15−/− mice like thioglycollate (unpublished observation). These findings support the importance of Alox15/PPARγ pathway in development of experimental hypertension.

Data presented in this study indicate that the absence of a functional macrophage-localized PPARγ prevents experimental hypertension from developing (Fig. 6). Paradoxically, synthetic PPARγ agonists such as thiazolidinedione (TZD) class of drugs are known to attenuate hypertension (22, 24), although the mechanism is not completely understood (25). The beneficial effect of TZD drugs could result from their anti-inflammatory properties, which occur even in the absence of the PPARγ (27). It can also be partially explained through a direct vascular effect mediated by endothelial PPARγ, which is protective against endothelial dysfunction (25). The effects of TZD drugs are generalized and affect most tissues including macrophage PPARγ. This lack of tissue specificity may lead to multiple counteracting mechanisms on blood pressure. In contrast, our results indicate that intraperitoneal thioglycollate injection selectively upregulates the macrophage PPARγ without affecting other organs, including aorta and kidney. Intraperitoneal injection of thioglycollate results in localized peritonitis, a process accompanied by macrophage influx into the peritoneal cavity. Possibly, this process is not so much a selective upregulation as a localized effect of thioglycollate on the immune cell population of the peritoneal cavity. Nevertheless, this local upregulation of macrophage PPARγ is sufficient to convey the development of l-NAME-induced hypertension (Fig. 6).

In summary, this study indicates a pivotal role for the macrophage Alox15/PPARγ pathway in development of l-NAME-induced hypertension. Thioglycollate-induced upregulation of macrophage PPARγ bypasses the missing component in Alox15−/− macrophages, allowing the Alox15-deficient mice to become hypertensive upon l-NAME treatment. The hypertension is blocked by a selective PPARγ antagonist. Our results indicate a need for a cell-specific approach when studying the role of PPARγ in hypertension.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: T.K., K.M.G., and W.B.C. conception and design of research; T.K. and C.C. performed experiments; T.K. and C.C. analyzed data; T.K., K.M.G., and W.B.C. interpreted results of experiments; T.K. prepared figures; T.K. drafted manuscript; T.K., K.M.G., and W.B.C. edited and revised manuscript; W.B.C. approved final version of manuscript.

REFERENCES


