Role of inducible nitric oxide synthase in the regulation of VCAM-1 expression in gut inflammation

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Kawachi, Shigeyuki, Adam Cockrell, F. Stephen Laroux, Laura Gray, D. Neil Granger, Henri C. van der Heyde, and Matthew B. Grisham. Role of inducible nitric oxide synthase in the regulation of VCAM-1 expression in gut inflammation. Am. J. Physiol. 277 (Gastrointest. Liver Physiol. 40): G572–G576, 1999.—The objectives of this study were to assess the role of the inducible isoform of nitric oxide synthase (iNOS) on vascular cell adhesion molecule 1 (VCAM-1) expression in vivo in an acute model of inflammation induced in iNOS-deficient (iNOS−/−) mice and compare these data to those obtained by pharmacological inhibition of iNOS in a CD4+ T lymphocyte-dependent model of chronic colitis. VCAM-1 expression was quantified in vivo using the dual radiolabeled monoclonal antibody technique. We found that intraperitoneal injection of 10 µg/kg tumor necrosis factor-α (TNF-α) enhanced VCAM-1 expression by approximately twofold in the colon, cecum, and stomach but not small intestine in iNOS−/− mice compared with TNF-α-injected wild-type mice. Injection of wild-type mice with 25 µg/kg TNF-α further enhanced VCAM-1 expression by approximately twofold compared with wild-type mice injected with 10 µg/kg TNF-α; however, VCAM-1 expression was not further enhanced in any gastrointestinal organ system in iNOS−/− mice. In a second series of experiments, we found that continuous inhibition of iNOS using oral administration of Nω-iminoethyl-L-lysine did not alter the enhanced levels of VCAM-1 expression in the colon nor did it alter the severity of colonic inflammation in SCID mice reconstituted with CD4+, CD45RBhigh T cells. We conclude that iNOS may regulate VCAM-1 expression in acute inflammation; however, this effect is modest and tissue specific and occurs only when VCAM-1 expression is submaximal. iNOS does not appear to modulate VCAM-1 expression in an immune model of chronic colitis.

neutrophils; nuclear factor-κB; endothelium

THE INFILTRATION of leukocytes into inflamed tissues results from the adhesive interactions between leukocytes and endothelial cells within the postcapillary venules (6, 7). These adhesive interactions are regulated in an orderly fashion by sequential activation of different families of membrane adherence receptors on leukocytes and endothelial cells. One such endothelial cell adhesion molecule (ECAM) is vascular cell adhesion molecule 1 (VCAM-1). This member of the immunoglobulin supergene family is a redox-sensitive ECAM whose expression is enhanced by a variety of different bacterial products, cytokines, and oxidants (2, 18). It is thought that VCAM-1 plays an important role in adhesion and recruitment of mononuclear leukocytes (e.g., monocytes, lymphocytes) during times of chronic inflammation (2, 18). Several recent studies suggest that exogenous nitric oxide (NO) donors may downregulate cytokine-induced VCAM-1 expression in cultured endothelial cells in vitro (8, 14, 20, 21, 24–27). The mechanisms by which exogenous NO exerts this anti-inflammatory effect have not been fully delineated; however, it is thought that NO inhibits the activation of nuclear factor-κB (NF-κB) by enhancing expression and/or stabilization of its inhibitor IκB and/or by inhibiting the binding of the p50/p65 heterodimer to its consensus sequence in the promoter/enhancer region upstream of VCAM-1 (8, 20, 21, 24, 25, 27). Because chronic gut inflammation is associated with the upregulation of the inducible isoform of nitric oxide synthase (iNOS) and the sustained overproduction of NO as well as enhanced infiltration of mononuclear leukocytes (3, 9), we wished to assess the effects of iNOS inhibition on VCAM-1 expression in vivo in an acute model of inflammation induced in iNOS-deficient mice (iNOS−/−) and compare these data to those obtained in an immune-based model of chronic colitis.

MATERIALS AND METHODS

Animals. Male C57BL/6 mice (n = 25) and iNOS−/− mice (n = 13) weighing 20–30 g were obtained from Harlan Sprague Dawley (Frederick, MD) and Jackson Laboratory (Bar Harbor, ME), respectively. Functionally inactive iNOS was produced by disruption of the iNOS gene by insertion of the neomycin gene into the calmodulin domain of the iNOS gene (16) and confirmed using a mouse model of lipopolysaccharide (LPS)-induced upregulation of iNOS-derived NO (10). Female C.B-17 and congenic C.B-17 (scid/scid) mice between 5 and 7 wk of age were obtained from Taconic Farms (Germantown, NY) and used for the colitis studies described in T cell-mediated chronic inflammation.

Tumor necrosis factor-α-induced acute inflammation. An acute animal model of inflammation was produced by intraperitoneal injection of recombinant murine tumor necrosis factor-α (TNF-α; 10 µg/kg and 25 µg/kg) (R&D Systems, Minneapolis, MN) as previously described (12). This model induces an acute inflammatory response in mice characterized by increased ECAM expression in different organ systems, including stomach, small intestine, cecum, and colon 5 h after TNF-α administration (12). VCAM-1 expression was quantified in the different tissues using the dual radiolabeled monoclonal antibody method of Komatsu et al. (15).

iNOS message was assessed in colons using RT-PCR in which total RNA was isolated, and CDNA was synthesized using reverse transcriptase and then amplified using the following primers: sense primer, 5′-AGAGTTTGACCAGAGCCACCC-3′; antisense primer, 5′-AAGACCAGAGGCAGCA
colonic inflammation was produced by transfer of CD4+ T cells, which did not develop signs of clinical disease.

A chronic model of colonic inflammation was produced by transfer of CD4+ T cells from healthy donor mice into immunodeficient SCID mice (5, 17). This procedure induces a chronic colitis 8 wk following reconstitution with this lymphocyte subset, whereas injection of SCID mice with PBS or CD4−, CD45RBlow T cells does not produce disease (5, 17). This model of colitis is characterized by colonic mucosal thickening, epithelial cell hyperplasia, erosions, and crypt abscesses as well as enhanced iNOS and VCAM-1 expression (5, 17).

Male C.B-17 SCID mice at the age of 5–7 wk were injected (intraperitoneally) with either PBS or CD4+ 5 × 105 CD45RBhigh or CD45RBlow T cells suspended in 500 µl of PBS. Body weights and fecal status were followed and recorded weekly from the time of the injection. At 4–6 wk following reconstitution with CD45RBhigh T cells, mice began to lose body weight and developed loose stools. At 8 wk following reconstitution, when animals lost ~10% of their initial body weight, ECAM quantification was performed in these animals as well as in SCID mice injected with PBS or CD45RBlow T cells, which did not develop signs of clinical disease.

The role of iNOS in regulating VCAM-1 expression was assessed in this model of chronic gut inflammation using the selective iNOS inhibitor Nω-iminoethyl-L-lysine (L-NIL) at a dose of 25 mg·kg−1·day−1 po beginning on week 4 when inflammation is minimal or absent and continuing until week 8 when colitis is maximal. Preliminary studies demonstrated that this dose of oral L-NIL inhibits LPS-induced production of NO-derived nitrate and nitrite by ~80% (326 ± 3 vs. 91 ± 37 µM for vehicle and L-NIL-treated mice injected with LPS, respectively). After 4 wk of L-NIL treatment, animals were anesthetized and colonic VCAM-1 expression was quantified as described above. Macroscopic inflammation was scored as described previously (4).

RESULTS

We found that a single intraperitoneal injection of 10 µg/kg TNF-α significantly enhanced VCAM-1 expression in the vasculature of the colon only; however, trends for increased expression of VCAM-1 were observed in the cecum, small intestine, and stomach in wild-type mice compared with their saline-injected controls (Fig. 1). This TNF-α-enhanced VCAM-1 expression in the colon was associated with the upregulation of colonic iNOS message (Fig. 2). Intraperitoneal injection of 10 µg/kg TNF-α also significantly enhanced VCAM-1 expression in the pancreas, mesentery, liver, and skeletal muscle; however, trends for increased expression were observed in virtually all tissue analyzed (Table 1). When these same experiments were performed in iNOS−/− mice, colonic, cecal, and stomach but not small intestinal VCAM-1 expression was enhanced further by ~70–100% in iNOS−/− mice given 10 µg/kg compared with the TNF-α-injected wild-type controls, suggesting that the potential regulatory role of iNOS on VCAM-1 expression was tissue specific. Enhanced VCAM-1 expression in iNOS−/− mice was not significantly enhanced by 10.2 ± 3.5 µg/kg TNF-α (Fig. 2).

![Fig. 1. Vascular cell adhesion molecule 1 (VCAM-1) expression in the vasculature of the gastrointestinal tract of wild-type (wt) and iNOS−/− mice injected (intraperitoneally) with tumor necrosis factor-α (TNF-α; 10 or 25 µg/kg).](http://ajpgi.physiology.org/)

![Fig. 2. RT-PCR determination of iNOS message in colons from wild-type mice injected with either saline or 10 µg/kg TNF-α.](http://ajpgi.physiology.org/)
Table 1. VCAM-1 expression in different tissues of wild-type and iNOS−/− mice

<table>
<thead>
<tr>
<th>Constitutive</th>
<th>Lung</th>
<th>Heart</th>
<th>Pancreas</th>
<th>Mesentery</th>
<th>Kidney</th>
<th>Liver</th>
<th>Spleen</th>
<th>Brain</th>
<th>Muscle</th>
<th>Skin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>305 ± 46</td>
<td>178 ± 25</td>
<td>106 ± 11</td>
<td>96 ± 5</td>
<td>1,416 ± 59</td>
<td>417 ± 33</td>
<td>10,960 ± 503</td>
<td>36 ± 2</td>
<td>50 ± 3</td>
<td>92 ± 3</td>
</tr>
<tr>
<td>TNF-α (10 µg/kg) induced</td>
<td>308 ± 4</td>
<td>138 ± 4</td>
<td>97 ± 4</td>
<td>106 ± 9</td>
<td>1,408 ± 10</td>
<td>504 ± 44</td>
<td>7,097 ± 431</td>
<td>35 ± 1</td>
<td>45 ± 3</td>
<td>85 ± 9</td>
</tr>
</tbody>
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Wild-type | 556 ± 105 | 470 ± 129 | 189 ± 26* | 481 ± 106* | 1,545 ± 157 | 1,680 ± 270* | 13,242 ± 1,208 | 109 ± 29 | 128 ± 10* | 79 ± 26 |
| iNOS−/− | 1,187 ± 56** | 561 ± 23* | 279 ± 3* | 248 ± 18 | 2,678 ± 114** | 2,686 ± 232** | 10,194 ± 689 | 122 ± 5* | 149 ± 3* | 171 ± 7** |

Wild-type | 1,191 ± 93* | 794 ± 91* | 324 ± 13* | 418 ± 28* | 2,252 ± 266* | 3,362 ± 261* | 10,934 ± 903 | 116 ± 3 | 149 ± 9* | 151 ± 10 |
| iNOS−/− | 1,635 ± 113** | 748 ± 21 | 331 ± 11* | 409 ± 20 | 2,559 ± 68* | 4,136 ± 99* | 6,154 ± 154 | 132 ± 3* | 175 ± 6* | 151 ± 7** |

Values (in ng monoclonal antibody/g tissue) are means ± SE. iNOS, inducible nitric oxide synthase; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1. *P < 0.05 vs. constitutive VCAM-1 expression for wild-type or iNOS−/− mice; †P < 0.05 vs. wild-type mice.

also observed in lung, pancreas, kidney, liver, and skin compared with that observed in the TNF-α-injected wild-type counterparts (Table 1). Increasing the dose of TNF-α to 25 µg/kg further enhanced VCAM-1 expression in the colon, cecum, small bowel, and stomach by approximately twofold as well as in other tissues (Table 1) compared with wild-type mice injected with 10 µg/kg TNF-α. Interestingly, when TNF-α was administered to iNOS−/− mice, no further increase in VCAM-1 expression was observed in most tissues compared with the corresponding TNF-α-injected wild-type controls (Fig. 1). We did, however, observe a further enhancement in VCAM-1 expression in the lung of iNOS−/− mice injected with this same amount of TNF-α (Table 1).

In a second series of experiments, we quantified colonic VCAM-1 expression in mice with chronic colitis induced by reconstitution of SCID mice with CD45RBhigh T cells (20, 21). We found that colonic VCAM-1 expression increased approximately fourfold in SCID mice reconstituted with CD45RBhigh T cells compared with their PBS-injected or CD45RBlow-reconstituted SCID controls (Fig. 3). This enhanced VCAM-1 expression correlated well with the onset of chronic inflammation and iNOS expression in the colon as previously described (21). Continuous inhibition of iNOS in this T cell model of chronic colitis using an oral dose of l-NIL known to inhibit iNOS in vivo (see MATERIALS AND METHODS) did not alter the enhanced levels of VCAM-1 expression in the colon nor did it alter macroscopic inflammation (Fig. 3).

**DISCUSSION**

There is increasing evidence to suggest that the free radical NO is very effective at modulating leukocyte-endothelial cell interactions in vitro and in vivo. For example, it has been demonstrated that NO attenuates the adhesion and recruitment of leukocytes in postcapillary venules exposed to different acute inflammatory stimuli such as ischemia and reperfusion, oxidized...
low-density lipoproteins, or reactive oxygen metabolites (6–8). An anti-adhesive role for NO is also supported by the observation that inhibitors of endothelial NOS elicit the recruitment of adherent leukocytes in postcapillary venules (8). The precise mechanisms responsible for this “anti-inflammatory” effect of NO are not entirely clear; however, several studies using cultured endothelial cells suggest that NO inhibits NF-κB activation by virtue of its ability to induce and or stabilize the expression of IκB and/or by inhibiting binding of the p50/p65 heterodimer to the enhancer-promoter region upstream of the VCAM-1 gene (21, 24, 25). Although several studies from our laboratory as well as others have shown potent antiadhesive activity in vitro and in acute inflammation in vivo, there is little or no evidence indicating that NO may modulate either positively or negatively ECAM expression in chronic inflammation in vivo. Indeed, numerous studies have demonstrated that chronic gut inflammation is associated with the infiltration of large numbers of mononuclear leukocytes coincident with the upregulation of iNOS and sustained overproduction of NO (1, 4, 11, 13, 19, 22). Furthermore, several studies have shown that certain NOS inhibitors possess anti-inflammatory activity in different models of inflammatory bowel disease (1, 4, 11, 13, 19, 22). These data suggest that iNOS-derived NO may play little or no role in modulating the chronic inflammatory response in vivo. Therefore, we wished to determine what role, if any, iNOS played in modulating VCAM-1 expression in a cytokine-induced model of acute inflammation vs. an immune-based model of chronic colitis.

We found that intraperitoneal administration of TNF-α produced a dose-dependent increase in VCAM-1 expression in vivo in several different tissues. The increase in vascular surface expression of VCAM-1 induced by 10 µg/kg TNF-α correlated well with increases in iNOS message in tissues such as the colon (Fig. 2). This is not surprising in view of the fact that both VCAM-1 and iNOS expression are induced by TNF-α via activation of NF-κB (3). Using 10 µg/kg TNF-α, we also observed a further twofold increase in VCAM-1 expression in several different tissues, including the colon, cecum, and stomach as well as the lung, pancreas, kidney, liver, and skin in the iNOS−/− mice compared with wild-type mice injected with the same dose of this cytokine (Fig. 1 and Table 1). An equally interesting observation was the lack of further VCAM-1 expression in a similar number of tissues from iNOS−/− mice injected with 10 µg/kg TNF-α compared with their wild-type controls injected with the same amount of the cytokine (Fig. 1 and Table 1). The reasons for this apparent tissue specificity in response to TNF-α are not entirely clear at the present time. One possibility may be that the physical location of iNOS in the various tissues in relation to the postcapillary venules dictates whether iNOS-derived NO acts to modulate VCAM-1 expression. For example, if iNOS is localized within or in close proximity to the postcapillary venules, one would predict a modulatory role for iNOS-derived NO. This possibility does not appear likely, since one would have to envision a completely different localization of iNOS in the small bowel vs. the rest of the gastrointestinal tract.

Previous studies from our laboratory have determined that 10 µg/kg TNF-α produces submaximal expression of VCAM-1 in most tissue (12, 15). Therefore, we wished to assess the modulatory role of iNOS in this same model when the dose of TNF-α was increased to 25 µg/kg, a dose previously shown to induce maximal VCAM-1 expression in the mouse (12, 15). We found that increasing the dose of TNF-α to 25 µg/kg enhanced VCAM-1 expression in most tissues by approximately twofold compared with wild-type mice injected with 10 µg/kg of the same cytokine (Fig. 1 and Table 1). Unexpectedly, this increase was not further enhanced in most tissues of iNOS−/− mice as had been shown for the 10 µg/kg dose (Fig. 1 and Table 1). The one exception was the lung, which responded with a further increase in VCAM-1 expression in iNOS−/− mice. Taken together, these data would suggest that iNOS-derived NO may modulate VCAM-1 expression in a tissue-specific manner. Furthermore, our data demonstrate that NO may not be an effective modulatory agent when VCAM-1 is fully expressed, i.e., when the levels of TNF-α are elevated to concentrations that may occur locally in more chronic models of inflammation such as inflammatory bowel disease.

To address this possibility directly, we assessed the effects of continuous iNOS inhibition on VCAM-1 expression in an immune-based model of chronic colitis in mice. We found that reconstitution of SCID mice with congenic CD4−, CD45RBhigh T cells but not with PBS nor with CD4−, CD45RBlow T cells produced clinical and histopathological signs of chronic colitis beginning 5–6 wk postreconstitution, which was associated with enhanced expression of iNOS and a fourfold increase in colonic VCAM-1 expression compared with PBS- or CD45RBlow-injected controls (Fig. 3). Continuous oral administration of the selective iNOS inhibitor L-NIL beginning 4 wk postreconstitution and continuing for an additional 4 wk did not alter the enhanced levels of VCAM-1 expression (Fig. 3) nor did it affect the colonic inflammation scores of these mice (Fig. 3). These data suggest that iNOS-derived NO does not modulate VCAM-1 expression to any significant extent in vivo in this model of colitis. This is not surprising in view of the data described above demonstrating the loss of modulation of VCAM-1 by iNOS when TNF-α levels are elevated as little as 2.5-fold. It is well known that local concentrations of different cytokines (especially TNF-α) are enhanced in experimental models as well as in human inflammatory bowel disease (23).

In summary, our data suggest that iNOS-derived NO may modulate VCAM-1 expression during acute inflammation; however, this modest regulatory activity is tissue specific and occurs only within a narrow concentration range of TNF-α. Furthermore, our data suggest that iNOS does not appear to be involved in the regulation of VCAM-1 expression in an immune-based model of chronic colitis.
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