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Research report

Cellular mechanisms by which tumor necrosis factor-α produces disruption of the blood–brain barrier

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Abstract

The first goal of the present study was to determine the effect of tumor necrosis factor-α (TNF-α) on the permeability of the blood–brain barrier in vivo. The second goal of this study was to investigate cellular pathways responsible for changes in permeability of the blood–brain barrier in response to TNF-α. We examined the pial microcirculation in rats using intravital fluorescence microscopy. Permeability of the blood–brain barrier was quantitated by calculating the clearance of fluorescent-labeled dextran (mol. wt=10,000; FITC–dextran-10K) during superfusion with vehicle, tumor necrosis factor (TNF-α; 10 ng/ml), TNF-α in the presence of an inhibitor of soluble guanylate cyclase (ODQ; 1.0 μM), and TNF-α in the presence of an inhibitor of protein tyrosine kinase (genistein; 10 μM). During superfusion with vehicle, clearance of FITC–dextran-10K from pial vessels remained relatively constant during the experimental period. In contrast, superfusion with TNF-α markedly increased clearance of FITC–dextran-10K from the cerebral microcirculation. Topical application of ODQ and genistein, significantly inhibited increases in permeability of the blood–brain barrier to FITC–dextran-10K during application of TNF-α. Thus, TNF-α increases the permeability of the blood–brain barrier to a moderately sized molecule via a mechanism which appears to involve activation of soluble guanylate cyclase and protein tyrosine kinase. In light of evidence suggesting that TNF-α production is increased during cerebrovascular trauma, we suggest that the findings of this study may contribute to our understanding of the pathogenesis of disruption of the blood–brain barrier during brain trauma and inflammation.

Keywords: FITC–dextran; Cerebral venule; Soluble guanylate cyclase; Tyrosine kinase; Genistein; ODQ

1. Introduction

The blood–brain barrier minimizes the entry of solutes into brain tissue. This restriction is accomplished by the presence of tight junctions between adjacent endothelial cells and a paucity of pinocytotic vesicles in cerebral arterioles, venules and capillaries [34]. Under various pathophysiologic conditions, the blood–brain barrier can be altered to allow solutes to enter the brain extracellular environment. However, cellular pathways which contribute to disruption of the blood–brain barrier during these conditions are not entirely clear. An understanding of cellular mechanisms which regulate the permeability of the blood–brain barrier could contribute to the development of new therapeutic approaches for the treatment of brain trauma and neurological diseases which are accompanied by an increase in permeability of the blood–brain barrier.

Tumor necrosis factor-α (TNF-α) is an inflammatory cytokine that is released in response to bacterial and viral infections, during neurological diseases, and during tissue trauma [8,32,35,41]. Many studies have shown that TNF-α increases the permeability of the peripheral circulation [20,21,23,45]. The mechanism for the increase in permeability of the peripheral circulation in response to TNF-α is not entirely clear, but may be related to an increase in leukocyte adhesion to the endothelium [20,45], an increase in the production of nitric oxide [6] and/or disruption of the endothelial glycocalyx [17]. Investigators also have examined the effects of TNF-α on the permeability of the blood–brain barrier. However, there is some discrepancy in findings regarding the effects of TNF-α on the blood–brain barrier. In vitro studies have shown that TNF-α either does not alter [10] or produces an increase [9,24] in transport of molecules across cerebral endothelium. In vivo studies have suggested that TNF-α does not alter [4,33,38], decreases [37] or increases the permeability of the blood–brain barrier [1,11,29,42]. Cellular mechanisms which

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account for an increase in permeability of the blood–brain barrier in response to TNF-α are not clear, but have been speculated to be related to an increase in the synthesis/release of cyclooxygenase products [9,22,42], stimulation of nitric oxide release [5,13], and/or an increase in the expression of adhesion molecules [18].

The overall goal of the present study was to begin to examine potential cellular mechanisms which account for disruption of the blood–brain barrier in vivo in response to TNF-α. First, we examined whether inhibition of soluble guanylate cyclase could influence disruption of the blood–brain barrier in response to TNF-α. Second, we examined whether inhibition of protein tyrosine kinase could influence disruption of the blood–brain barrier in response to application of TNF-α.

2. Methods

2.1. Preparation of animals

Male Wistar–Furth rats were anesthetized (Inactin; thiobutabarbital 100 mg/kg, i.p.) and a tracheotomy was performed. The rats were mechanically ventilated with room air and supplemental oxygen. A catheter was placed in the left femoral artery and vein for the measurement of systemic blood pressure and for injection of the intravascular tracer, fluorescein isothiocyanate dextran (mol. wt= 10,000; FITC–dextran-10K), respectively. All procedures were carried out following institutional IACUC approval and were within institutional guidelines.

To visualize the cerebral microcirculation, a cranial window was prepared over the left parietal cortex using methods we have described previously [27]. An incision was made in the skin to expose the skull. The skin was retracted with sutures and served as a 'well' for the suffusion fluid. An inlet and outlet port were made in the skin to allow for the constant flow of suffusate across the cerebral (pial) microcirculation. Finally, a craniotomy was performed, the dura was incised and the cerebral microcirculation was exposed. The suffusion fluid (artificial cerebrospinal fluid) was heated (37±1 °C), and bubbled continuously with 95% nitrogen and 5% carbon dioxide to maintain gases within normal limits. Blood gases were also monitored and maintained within normal limits. At the end of the experiment all anesthetized rats were killed with an intravenous injection of saturated potassium chloride.

2.2. Permeability of the blood–brain barrier

The permeability of the blood–brain barrier was evaluated by calculating the clearance of fluorescent tracer ($ml/s \times 10^{-6}$) by pial vessels as we have described previously [26–28]. The suffusate fluid was collected in glass test tubes with the aid of a fraction collector and we determined the concentration of FITC–dextran-10K in the suffusate fluid during topical application of vehicle (saline), or TNF-α in the absence or presence of ODQ and genistein. Arterial blood samples (approximately 60 μl/sample) were drawn at various intervals throughout the experiment. FITC–dextran-10K was given as a constant intravenous infusion (40 mg/ml at 0.065 ml/min). To quantitate the concentration of fluorescent tracer in the suffusate fluid and plasma samples, standard curves for concentration of FITC–dextran-10K versus percent transmission were obtained with a spectrophotofluorometer (Perkin–Elmer; Model LS30). The standards were prepared on a weight-per-volume basis. The suffusate concentration was used as background, a standard curve was generated for each experiment, and each curve was subjected to linear regression analysis. The percent transmission for unknown samples (suffusate and plasma) was measured on the spectrophotofluorometer, and the concentration was calculated from the standard curve. The clearance of FITC–dextran-10K was calculated by multiplying the ratio of suffusate-to-plasma concentration by the suffusate flow-rate [26–28].

2.3. Pial arteriolar diameter

The diameter of pial arterioles was measured on-line using a video image shearing device (model 908, Instrumentation for Physiology and Medicine, Inc.). We measured the diameter of the largest pial arteriole exposed by the craniotomy before and during application of vehicle, and TNF-α in the absence or presence of ODQ or genistein. In the present study, we report the diameter of pial arterioles immediately before application of vehicle or TNF-α in the absence or presence of ODQ or genistein, and at various intervals thereafter during a continuous topical application.

2.4. Experimental protocol

In the first group of rats ($n=6$) we examined the clearance of FITC–dextran-10K from the cerebral microcirculation during topical application of vehicle. Thus, 2 h following the preparation of the craniotomy, we started a continuous topical application of vehicle. One hour later, we started a continuous intravenous infusion of FITC–dextran-10K. Clearance of FITC–dextran-10K was determined for the next 120 min during suffusion with vehicle. Thus, the cerebral microcirculation was exposed to vehicle for a total of 3 h. We report the clearance of FITC–dextran-10K by the cerebral microcirculation at various time intervals after starting intravenous infusion of FITC–dextran-10K. In addition, we measured the diameter of pial arterioles before and at various time intervals during application of vehicle.

In a second group of rats ($n=5$) we examined the clearance of FITC–dextran-10K from the cerebral microcirculation during topical application of TNF-α (10

ng/ml). Thus, 2 h following preparation of the craniotomy, we started a continuous application of TNF-α. One hour later, we started a continuous intravenous infusion of FITC–dextran-10K. Clearance of FITC–dextran-10K was determined for the next 120 min during suffusion with TNF-α. Thus, the total exposure time of the cerebral microcirculation to TNF-α was 3 h. We report the clearance of FITC–dextran-10K by the cerebral microcirculation at various time intervals after starting intravenous infusion of FITC–dextran-10K. In addition, we measured the diameter of pial arterioles before and at various time intervals during application of TNF-α.

In a third group of rats ($n=6$) and fourth ($n=4$) group of rats we examined the effects of inhibition of soluble guanylate cyclase using ODQ (1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one; 1.0 μM) and inhibition of protein tyrosine kinase using genistein (10 μM) on TNF-α-induced increases in permeability of the blood–brain barrier. Thus, in these studies a similar protocol was followed as that described above with the exception that a continuous application of ODQ or genistein was applied to the cerebral microcirculation 30 min prior to application of TNF-α. The exposure time to TNF-α was 3 h and we report the clearance of FITC–dextran-10K by the cerebral microcirculation at various time intervals after starting intravenous infusion of FITC–dextran-10K. In addition, we measured the diameter of pial arterioles before application of ODQ or genistein, at various time intervals during application of ODQ and genistein in the absence of TNF-α, and at various time intervals during application of ODQ and genistein in the presence of TNF-α.

2.5. Statistical analysis

Analysis of variance with Fisher's test for significance was used to compare clearance of FITC–dextran-10K and the diameter of pial arterioles during suffusion with vehicle and TNF-α in the absence and presence of ODQ or genistein. Data is reported as means±S.E.Ms. A P-value of 0.05 or less was considered to be significant.

3. Results

3.1. Permeability of the blood–brain barrier

During suffusion with vehicle, the clearance of FITC–dextran-10K from the cerebral microcirculation increased during the first 60 min after starting intravenous infusion of FITC–dextran-10K, and then remained relatively constant for the remainder of the experimental period (Fig. 1).

During suffusion with TNF-α, the clearance of FITC–dextran-10K from the cerebral microcirculation was elevated at all time points examined after starting infusion of FITC–dextran-10K. In addition, unlike that observed while suffusing with vehicle, the clearance of FITC–dextran-10K continued to increase during the experimental period (Fig. 1). Thus, it appears that application of TNF-α to the cerebral microcirculation in vivo produces an increase in the permeability of the blood–brain barrier to a moderately sized molecule.

Next, we examined whether application of ODQ might influence disruption of the blood–brain barrier in response

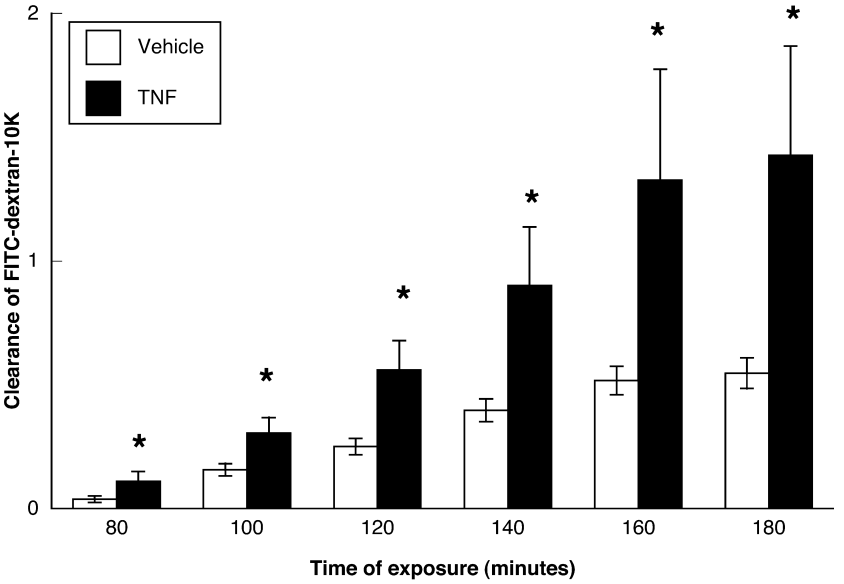

Fig. 1. Clearance of FITC–dextran-10K (ml/s×10^{-6}) at various time intervals during application of vehicle (open bars) and TNF-α (10 ng/ml; closed bars). Application of vehicle and TNF-α were started 60 min prior to infusion of FITC–dextran-10K. Values are means±S.E.Ms. *$P<0.05$ versus response while suffusing with vehicle.

to TNF-α. We found that a continuous topical application of ODQ significantly inhibited the increase in clearance of FITC–dextran-10K observed during suffusion with TNF-α (Fig. 2). In fact, there was no difference in the clearance of FITC–dextran-10K by the cerebral microcirculation during suffusion of vehicle and suffusion of TNF-α in the presence of ODQ (Figs. 1 and 2).

Finally, we examined whether application of genistein might influence disruption of the blood–brain barrier in response to TNF-α. We found that a continuous topical application of genistein significantly inhibited the increase in clearance of FITC–dextran-10K observed during suffusion with TNF-α (Fig. 3). Similar to that reported for ODQ, there was no difference in the clearance of FITC–dextran-10K by the cerebral microcirculation during suffusion of vehicle and suffusion of TNF-α in the presence of genistein (Figs. 1 and 3).

3.2. Arteriolar diameter

Application of vehicle did not alter the diameter of cerebral arterioles during the time course of the experiment (Fig. 4). Baseline diameter of pial arterioles was 52±4 μm before suffusing vehicle and remained relatively constant during the balance of the experimental period.

Similar to that observed while suffusing with vehicle, topical application of TNF-α (10 ng/ml) did not alter the diameter of pial arterioles (Fig. 4). Baseline diameter of pial arterioles was 45±3 μm before suffusing with TNF-α and remained near this diameter during the balance of the experimental period (Fig. 4).

Suffusion of the cerebral microcirculation with ODQ produced a modest, but significant, decrease in the diameter of pial arterioles (Fig. 5). Baseline diameter of pial arterioles was 49±3 μm before suffusing with ODQ and decreased to 42±2 μm 30 min during suffusion with ODQ ($P<0.05$). Suffusion with TNF-α was started following 30 min of suffusion with ODODQ. We found that suffusion of TNF-α, in the presence of ODQ, did not produce a change in the diameter of pial arterioles (Fig. 5). The diameter of pial arterioles was 42±2 μm while suffusing with ODQ, before suffusing TNF-α, and remained at this level during suffusion with TNF-α (Fig. 5).

Suffusion of the cerebral microcirculation with genistein did not alter the diameter of pial arterioles (Fig. 5). Baseline diameter of pial arterioles was 52±7 μm before suffusing genistein and remained relatively constant during the next 30 min (Fig. 5). Suffusion of TNF-α was started following 30 min of suffusion with genistein. We found that suffusion of TNF-α, in the presence of genistein, did not alter the diameter of pial arterioles (Fig. 5). The diameter of pial arterioles was 55±7 μm while suffusing with genistein, before suffusing TNF-α, and remained at this level during suffusion with TNF-α (Fig. 5).

4. Discussion

There are three main findings of the present study. First, suffusion of TNF-α over the cerebral microcirculation in vivo produces an increase in permeability of the blood–brain barrier. Second, increases in permeability of the

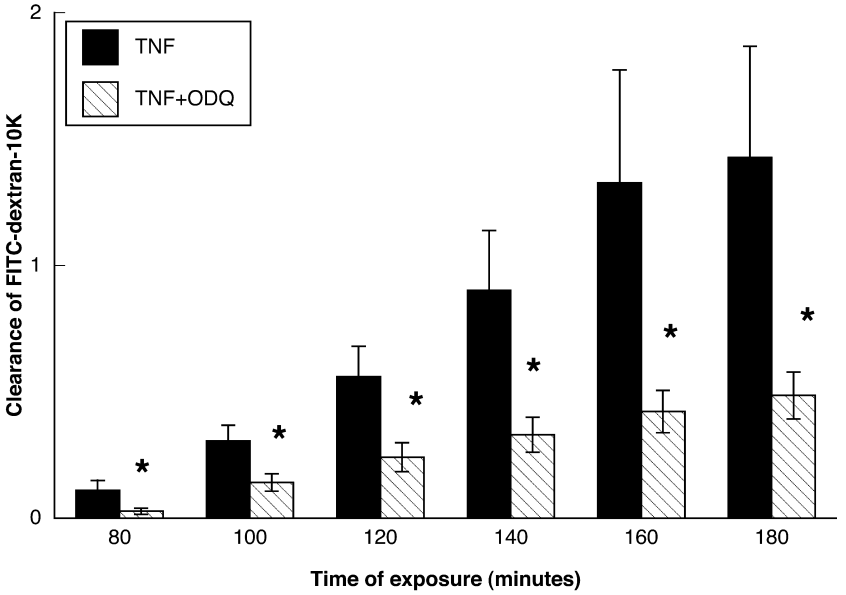

Fig. 2. Clearance of FITC–dextran-10K (ml/s×10^{-6}) at various time intervals during application of TNF-α (10 ng/ml) in the absence (closed bars) and presence (hatched bars) of ODQ (1.0 μM). Application of TNF-α was started 60 min prior to infusion of FITC–dextran-10K and application of ODQ was started 30 min prior to starting suffusion with TNF-α. Values are means±S.E.Ms. *$P<0.05$ versus response while suffusing with TNF-α.

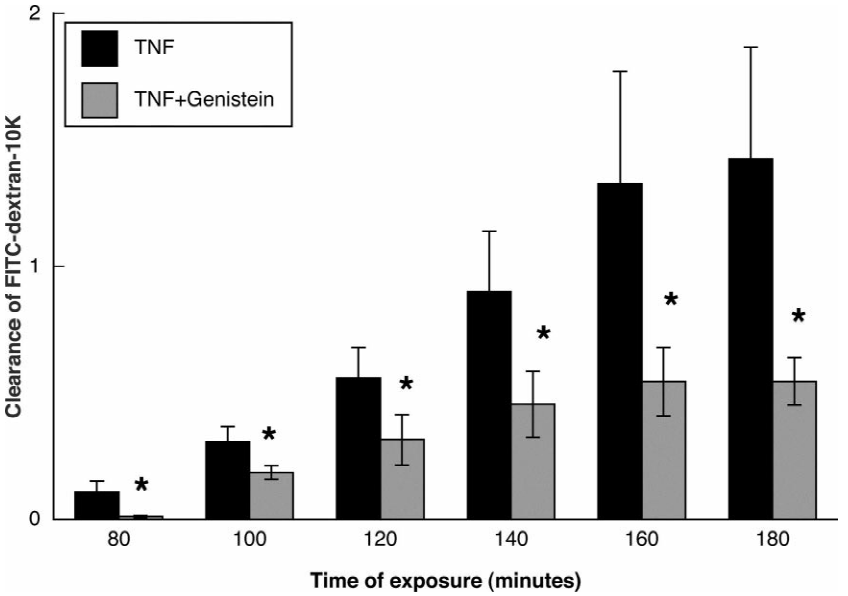

Fig. 3. Clearance of FITC–dextran-10K ($ml/s \times 10^{-6}$) at various time intervals during application of TNF-α (10 ng/ml) in the absence (closed bars) and presence (shaded bars) of genistein (10 μM). Application of TNF-α was started 60 min prior to infusion of FITC–dextran-10K and application of genistein was started 30 min prior to starting suffusion with TNF-α. Values are means±S.E.Ms. *$P<0.05$ versus response while suffusing with TNF-α.

blood–brain barrier in response to suffusion with TNF-α could be inhibited by ODQ, a specific inhibitor of soluble guanylate cyclase. Third, increases in permeability of the blood–brain barrier in response to suffusion with TNF-α could be inhibited by genistein, a specific inhibitor of tyrosine kinase. Thus, two scenarios are possible. First, it is possible that TNF-α activates specific receptors on the endothelium of cerebral blood vessels to activate tyrosine kinase. The activation of tyrosine kinase is followed by the release of a substance or substances to activate soluble guanylate cyclase, which in turn produces an increase in permeability of the blood–brain barrier. Second, it is also

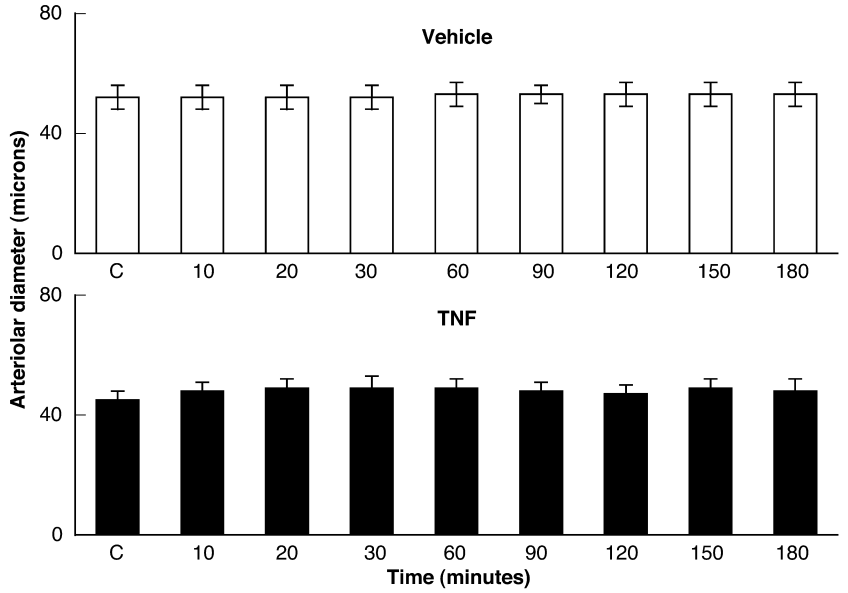

Fig. 4. Diameters of cerebral arterioles under control conditions (C) and at various time intervals following application of vehicle (upper panel; open bars) and TNF-α (lower panel; closed bars). Values are means±S.E.Ms.

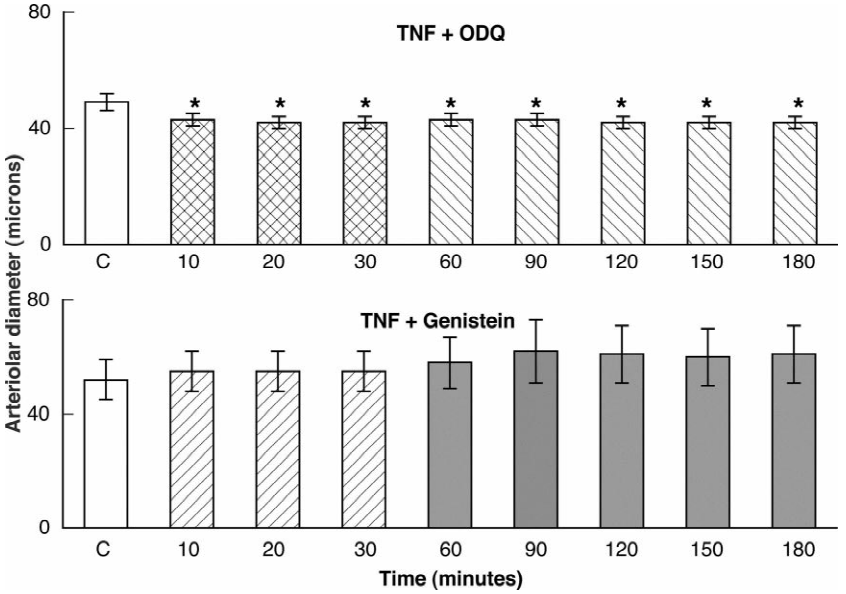

Fig. 5. (Upper panel) Diameter of cerebral arterioles under control conditions (C), during topical application of ODQ (1.0 μM; double hatched bars), and during topical application of ODQ in the presence of TNF-α (hatched bars). Values are means±S.E.Ms. *$P<0.05$ versus response before ODQ. (Lower panel) Diameter of cerebral arterioles under control conditions (C), during topical application of genistein (10 μM; hatched bars), and during topical application of genistein in the presence of TNF-α (shaded bars). Values are means±S.E.Ms.

possible that TNF-α activates guanylate cyclase which, in turn, activates tyrosine kinase to produce an increase in permeability of the blood–brain barrier.

4.1. Consideration of methods

We used ODQ to examine the role of activation of soluble guanylate cyclase on the permeability of the blood–brain barrier and on pial arteriolar diameter during suffusion with TNF-α. ODQ has been shown to be a selective inhibitor of soluble guanylate cyclase [16], and we [25] and others [12,14,31,40] have used ODQ to examine the role of activation of soluble guanylate cyclase in reactivity and permeability of the cerebral circulation in response to many agonists.

We used genistein to examine a potential role of activation of tyrosine kinase in permeability of the blood–brain barrier in response to TNF-α. Genistein has been shown to be a specific inhibitor of tyrosine kinase [2]. While several studies have shown that inhibition of protein tyrosine kinase using genistein can reduce agonist-induced changes in vascular diameter [30] and agonist-induced increases in peripheral microvascular permeability [15,19,43,44], no studies that we are aware of have examined the effects of inhibition of tyrosine kinase using genistein agonist-induced increases in permeability of the blood–brain barrier. Thus, we suggest that the use of ODQ and genistein are appropriate for examining the role of guanylate cyclase and tyrosine kinase, respectively, in agonist-induced changes in permeability of the blood–brain barrier.

4.2. Effect of TNF-α on arteriolar diameter

Few studies have examined the effects of TNF-α on vascular reactivity of the cerebral circulation. Brian and Faraci [7] found that topical application of TNF-α produced a marked dilatation of rat pial arterioles which could be inhibited by aminoguanidine and dexamethasone. A study by Angstwurm et al. [3] report that intracisternal injection of TNF-α produced a dose-related increase in cerebral blood flow in rats. Low doses of TNF-α (similar to that used in the present study), however, did not alter cerebral blood flow [3]. Megyeri et al. [29] found that TNF-α produced a dose-related constriction of pial arterioles in newborn pigs, while Shibata et al. [39] found that TNF-α produced dilatation of pial arterioles in newborn pigs. The discrepancy between these studies [29,39] appears to be related to the route of administration of TNF-α. In the present study, we found that topical application of TNF-α did not alter the diameter of pial arterioles in rats. The discrepancy between results of the present study and that of Brian and Faraci [7] may be related to the concentration of TNF-α. The study of Brian and Faraci [7] used a 10-fold higher concentration of TNF-α than that used in the present study.

4.3. Effect of TNF-α on the blood–brain barrier

Several studies have examined the effects of TNF-α on the permeability of isolated cerebral vascular endothelium. There is some discrepancy in findings regarding the effects of TNF-α on the in vitro blood–brain barrier. Descamps et al. [10] report that TNF-α did not alter the permeability of cultured bovine brain capillary endothelial cells to sucrose or inulin. However, others [9,24] have shown that TNF-α decreases electrical resistance of cultured rat brain microvascular endothelial cells [9] and an increase in the transport of various sized molecules across cultured bovine brain microvessel endothelial cell monolayers [24]. The discrepancy between these studies regarding the effects of TNF-α on the permeability of the in vitro blood–brain barrier remains elusive, but may related to the concentration and duration of exposure to TNF-α.

In addition to in vitro studies, others have examined the effects of TNF-α on the permeability of the blood–brain barrier using in vivo methodologies. Saija et al. [37] found that intracarotid injection of TNF-α produced a marked decrease in permeability of the blood–brain barrier to aminoisobutyric acid (AIB). Others [4,33,38] have reported that intracerebral injection of TNF-α does not alter the permeability of the blood–brain barrier in rats or mice. In contrast, Megyeri et al. [29] found that intracisternal injection of TNF-α in newborn swine produced a marked increase in permeability of the blood–brain barrier to small molecules (sodium fluorescein; mol. wt=376). Similarly, Abraham et al. [1] found that intracarotid injection of TNF-α produced an increase in the permeability of the blood–brain barrier to large (Evans blue–albumin) and small (sodium fluorescein) molecules in newborn pigs. Dickstein et al. [11] found that intraventricular injection of TNF-α in rats produced an increase in the efflux of radiolabeled albumin into the cerebrospinal fluid. Finally, Rosenberg et al. [36] report that intracerebral injection of TNF-α in rats produced an increase in the permeability of the blood–brain barrier to sucrose. Thus, several studies have shown that application of TNF-α to the cerebral circulation produces a marked increase in permeability of the blood–brain barrier. The discrepancy between these studies [1,11,29,36] and others [4,33,37,38] is not entirely clear, but may relate to the duration of exposure, the route of administration, and/or the dose of TNF-α used to examine the permeability of the blood–brain barrier. In the present study, we found that topical application of TNF-α produced a marked increase in the permeability of the blood–brain barrier in rats to a moderately sized tracer. The results of the present study extend that of previous studies by examining potential cellular mechanisms which account for increases in permeability of the blood–brain barrier during exposure to TNF-α.

While studies have suggested that TNF-α increases the permeability of the blood–brain barrier both in vitro and in vivo, cellular mechanisms responsible for this increase in permeability of the blood–brain barrier are less clear. An in vitro study [9] has shown that the effects of TNF-α on transendothelial electrical resistance could be inhibited by indomethacin, suggesting an important role for the activation of the cyclooxygenase pathway. Similarly, one in vivo study indirectly suggests a role for the cyclooxygenase pathway and TNF-α in change in permeability of the blood–brain barrier [42]. These investigators [42] found that increases in permeability of the blood–brain barrier to *E. coli* was related to TNF-α-induced activation of the cyclooxygenase pathway [42]. Intravenous injection of TNF-α and *E. coli* produced an increase in the expression of Cox-2 in cerebral arteries and treatment with NS398, a specific inhibitor of Cox-2, could reduce the increase in permeability of the blood–brain barrier in response to injection of *E. coli*. These investigators [42] concluded that TNF-α-induced stimulation of Cox-2 expression may play an important role in *E. coli*-induced changes in permeability of the blood–brain barrier. Unfortunately, these investigators did not directly examine the effects of TNF-α on the permeability of the blood–brain barrier and did not directly examine mechanisms by which TNF-α increases the permeability of the blood–brain barrier. Another study [36] found that TNF-α-induced increases in permeability of the blood–brain barrier to sucrose could be inhibited by batimastat, an inhibitor of metalloproteinases, suggesting a role for activation of proteolytic enzymes. Other studies, that did not examine the effects of TNF-α on the permeability of the blood–brain barrier, have shown that TNF-α stimulates nitric oxide release [5,13], activates cyclooxygenase enzymes (Cox-1 and Cox-2) [22], and increases the expression of adhesion molecules [18]. These findings led to the speculation that some or all of these pathways may contribute to increases in permeability of the blood–brain barrier in response to TNF-α. Thus, few studies have examined cellular mechanisms which account for changes in permeability of the blood–brain barrier in response to TNF-α. In the present study, we found that increases in permeability of the blood–brain barrier in response to TNF-α could be significantly inhibited by treatment with ODQ and genistein, suggesting an important role for soluble guanylate cyclase and tyrosine kinase, respectively, in TNF-α-induced changes in permeability of the blood–brain barrier.

In summary, we found that application of TNF-α to the cerebral microcirculation increases the permeability of the blood–brain barrier, but does not alter the diameter of cerebral resistance arterioles. Further, the increase in permeability of the blood–brain barrier in response to TNF-α could be inhibited by application of a specific inhibitor of guanylate cyclase, and a specific inhibitor of protein tyrosine kinase. Based upon these findings, we suggest that TNF-α activates specific receptors on the endothelium of cerebral blood vessels to stimulate tyrosine kinase. Activation of tyrosine kinase stimulates the release of a substance, presumably nitric oxide, which activates

soluble guanylate cyclase, which in turn produces an increase in permeability of the blood–brain barrier. Since TNF-α is released into the cerebrospinal fluid in response to bacterial and viral infections, during neurological disease states, and during brain trauma, we suggest that our findings have important implications for the pathogenesis of disruption of the blood–brain barrier during cerebrovascular abnormalities.

Acknowledgements

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