Methotrexate potentiates bradykinin-induced increase in macromolecular efflux from the hamster oral mucosa

XIAO-PEI GAO AND ISRAEL RUBINSTEIN
Department of Medicine, University of Illinois at Chicago, and the West Side Department of Veterans Affairs Medical Center, Chicago, Illinois 60612

Gao, Xiao-Pei, and Israel Rubinstein. Methotrexate potentiates bradykinin-induced increase in macromolecular efflux from the hamster oral mucosa. Am. J. Physiol. 273 (Regulatory Integrative Comp. Physiol. 42): R1254–R1262, 1997.—The purpose of this study was to determine whether methotrexate modulates bradykinin-induced increase in macromolecular efflux from the in situ oral mucosa and whether this response is mediated by the L-arginine/nitric oxide biosynthetic pathway. Using intravital microscopy, we found that suffusion of methotrexate alone onto the hamster cheek pouch had no significant effects on leaky site formation and increase in clearance of fluorescein isothiocyanate-labeled dextran (molecular mass, 70 kDa). However, methotrexate significantly potentiated bradykinin-induced responses (P < 0.05). These effects were associated with significant increases in nitrites concentration and guanosine 3′,5′-cyclic monophosphate-like immunoreactivity in the suffusate and were abrogated by Nω-nitro-L-arginine methyl ester (L-NAME) but not Nω-nitro-D-arginine methyl ester (D-NAME). L-Arginine, but not D-arginine, abolished L-NAME-induced responses. ZnCl₂ and indomethacin had no significant effects on methotrexate-induced responses. Methotrexate had no significant effects on adenosine- and ionomycin-induced increases in macromolecular efflux. Collectively, these data indicate that methotrexate amplifies bradykinin-induced increase in macromolecular efflux from the in situ oral mucosa in a specific, receptor- and L-arginine/nitric oxide biosynthetic pathway-dependent fashion.

Preparation of Animals

Adult male golden Syrian hamsters weighing 129 ± 1 g (n = 76) were anesthetized with pentobarbital sodium (6 mg/100 g body wt ip). A tracheotomy was performed to facilitate spontaneous breathing. A femoral vein was cannulated to inject the intravascular tracer, fluorescein isothiocyanate-labeled dextran (FITC-dextran); molecular mass 70 kDa; 40 mg/100 g body wt dissolved in 1.0 ml normal saline and administered over 1 min) and supplemental anesthesia (2–4 mg/100 g body wt−1·h−1). A femoral artery was cannulated to obtain arterial blood samples and to monitor arterial blood pressure, which did not change significantly during the experiments. Body temperature was kept constant (37–38°C) throughout the experiment using a heating pad.

To visualize the microcirculation of the cheek pouch, we used a method previously described in our laboratory (11–14, 27, 28, 34, 39). Briefly, the left cheek pouch was spread gently over a small plastic base plate, and an incision was made in the outer skin to expose the cheek pouch membrane. The avascular connective tissue layer was removed, and a plastic chamber was positioned over the base plate and secured in place by suturing the skin around the upper chamber. This chamber contained the suffusion fluid. This arrangement forms a triple-layered complex: the base plate, the upper chamber, and the cheek pouch membrane exposed between the two plates. After these initial procedures, the hamster was transferred to a heated microscope stage. The chamber was connected to a reservoir containing warmed bicarbonate buffer (37–38°C) composed (in mM) of 131.9 NaCl, 2.95 KCl, 1.48 CaCl₂, 0.76 MgCl₂, and 11.87 NaHCO₃, which allowed continuous suffusion of the cheek pouch. The buffer was bubbled continuously with 95% N₂-5% CO₂ (pH 7.4). The chamber was also connected via a three-way valve to an infusion pump (Sage Instruments, Boston, MA) that allowed for the constant administration of drugs into the suffusate.

Determination of Clearance of Macromolecules

The cheek pouch microcirculation was visualized with an Olympus microscope (Jacobs Instruments, Shawnee Mission, Kansas) by using a 10× objective and a 25× long distance objective. The chamber was illuminated with a 100-W HBO mercury arc lamp (Sage Instruments, Boston, MA). The fluorescent images were viewed on a monitor (Olympus, Tokyo, Japan) and photographed with a high-speed film (Kodak, Rochester, NY).
The phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (1.0 mM) was added to samples to avoid possible interference by changes in phosphodiesterase activity during application of test compounds and to facilitate cGMP accumulation. Optical density was read at 450 nm within 30 min at room temperature using a thermoregulated ELISA microplate reader (Molecular Devices). The sensitivity of the assay is 14 pg/ml, and cross-reactivity with adenosine 3',5'-cyclic monophosphate is <0.00008%.

Experimental Protocols

Effects of methotrexate on bradykinin-induced responses. The purpose of these studies was to determine whether methotrexate potentiates bradykinin-induced leaky site formation and increase in clearance of FITC-dextran from the cheek pouch. After bicarbonate buffer was suffused for 30 min (equilibration period), FITC-dextran was injected intravenously, and the number of leaky sites and clearance of FITC-dextran were determined for 30 min. Then, two concentrations of bradykinin (0.25 and 0.5 µM) were suffused onto the cheek pouch in an arbitrary order. Each concentration was suffused for 5 min (11, 12, 39). The number of leaky sites was determined every minute for 7 min, and at 5-min intervals for 45 min thereafter. Clearance of FITC-dextran was determined before and every 5 min for 45 min. The time interval between subsequent suffusions of bradykinin was at least 30 min (11, 12, 15, 39). Once suffusion of bradykinin was stopped and the number of leaky sites returned to baseline, methotrexate (5 mg/kg) was suffused for 30 min at a flow rate of 2 ml/min (final concentration in suffusate, 2.3 × 10⁻⁵ M), and suffusion of bradykinin was repeated. The number of leaky sites and clearance of FITC-dextran were determined during each intervention. In preliminary studies, we determined that repeated suffusions of bradykinin (0.25 and 0.5 µM) before and after suffusion of saline (vehicle) for 30 min were associated with reproducible results. In addition, suffusion of saline for the entire duration of the experiment was not associated with visible leaky site formation and increase in clearance of FITC-dextran. The concentrations of bradykinin and methotrexate used in these studies were based on previous studies in our laboratory and reports in the literature (2, 11, 12, 15, 17, 19, 20, 39).

Effects of L-NAME. The purpose of these studies was to determine whether the L-arginine/NO biosynthetic pathway mediates methotrexate potentiation of bradykinin-induced responses. After bicarbonate buffer was suffused for 30 min (equilibration period), FITC-dextran was injected intravenously, and the number of leaky sites and clearance of FITC-dextran were determined for 30 min. Then, two concentrations of bradykinin (0.25 and 0.5 µM) were suffused as outlined above. Once suffusion of bradykinin was stopped and the number of leaky sites returned to baseline, methotrexate (5 mg/kg) was suffused either in the absence or presence of N⁵-nitro-L-arginine methyl ester (l-NAME; 100 µM), an NO synthase inhibitor (11, 20, 27), or L-arginine (1 mM), the substrate for NO synthase (27, 29); l-NAME (100 µM) and methotrexate (5 mg/kg); or d-arginine (1 mM), L-NAME (100 µM), and methotrexate (5 mg/kg) for 30 min. The number of leaky sites and clearance of FITC-dextran were determined during each intervention. In preliminary studies, we determined that suffusion of l-NAME, d-NAME, L-arginine, and d-arginine alone for 30 min was not associated with visible leaky site formation and increase in clear-

Nitrites Assay

The concentration of nitrites in the suffusate was determined by a modified Griess reaction as previously described (31). Briefly, samples (1 ml) of the suffusate were incubated with Escherichia coli nitrate reductase (0.5 U/ml) at room temperature for 10 min to convert nitrites to nitric oxide. Thereafter, concentration of nitric oxide was measured in duplicate by mixing each sample with an equal volume (400 µl) of the Griess reagent. The mixture was incubated at room temperature for 10 min, and absorbency was then measured at 540 nm using a spectrophotometer (SpectraMax 340; Molecular Devices, Palo Alto, CA). The concentration of nitrites in each sample (expressed as µM) was determined from a standard curve obtained using known concentrations of NaNO₂ and NaNO₃ in distilled water. Nitrites concentration in buffer was subtracted from experimental values.

cGMP Assay

The concentration of guanosine 3',5'-cyclic monophosphate (cGMP)-like immunoreactivity in the suffusate was determined in duplicate using a commercial cGMP enzyme-linked immunosorbent assay (ELISA) kit (Amersham, Arlington Heights, IL) according to the manufacturer’s specifications.
ance of FITC-dextran. The concentrations of l-NAME, d-
NAME, l-arginine, and d-arginine used in these studies were
based on previous studies in our laboratory and reports in the
literature (20, 27, 29, 31, 34).

In another series of experiments, the suffusate was col-
collected into preciliated polypolypropylene test tubes during the last
5 min of 30-min suffusion period of saline (control), methotrex-
ate (5 mg/kg), and l-NAME (100 µM) alone. The suffusate was also
collected during 5-min suffusion of bradykinin (0.5 µM) alone,
bradykinin (0.5 µM) after suffusion of methotrexate (5
mg/kg) for 30 min, and bradykinin (0.5 µM) after suffusion of
methotrexate (5 mg/kg) and l-NAME (100 µM) for 30 min. Samples
were immediately snap-frozen in liquid nitrogen and stored at −70°C until used for determination of nitrites concentra-
tion and cGMP-like immunoreactivity.

Effects of ZnCl2. The purpose of these studies was to
determine whether methotrexate potentiation of bradykinin-
induced increase in macromolecular efflux is partly related to
chelation of the zinc moiety in catalytic domains of metalloen-
yzmes, such as angiotensin I-converting enzyme and neutral
endopeptidase 24.11, that cleave and inactivate bradykinin in the
cheek pouch (5, 16, 26, 36, 39). After bicarbonate buffer was suffused for 30 min (equilibration period), FITC-dextran
was injected intravenously, and the number of leaky sites and
clearance of FITC-dextran were determined for 30 min. Thereafter, bradykinin (0.5 µM) was suffused for 5 min before
and after ZnCl2 was suffused (100 µM) for 30 min. In another
group of animals, bradykinin (0.5 µM) was suffused for 5 min before and after suffusion of methotrexate (5 mg/kg) alone
or methotrexate (5 mg/kg) with ZnCl2 (100 µM) for 30 min. The
number of leaky sites and clearance of FITC-dextran were
determined during each intervention. In preliminary studies, we
determined that suffusion of ZnCl2 (100 µM) alone for 30
min was not associated with visible leaky site formation and
increase in clearance of FITC-dextran. The concentration of
ZnCl2 used in these studies was based on preliminary studies
and a previous report in the literature (16).

Effects of indomethacin. The purpose of these studies was
to determine whether products released through the cyclooxy-
genase pathway of arachidonic acid metabolism mediate, in
part, the potentiating effects of methotrexate on bradykinin-
induced increase in clearance of FITC-dextran from the cheek pouch (5, 16, 26, 36, 39). After bicarbonate buffer was suffused for 30 min (equilibration period), FITC-dextran was injected intravenously, and the number of leaky sites and clearance of FITC-dextran were determined for 30 min. Thereafter, bradykinin (0.5 µM) was suffused for 5 min before and after methotrexate was suffused (5 mg/kg) for 30 min. Then, indomethacin (10 mg/kg) was injected intravenously over a 30-min period using an infusion pump (final volume = 10
ml), and suffusion of methotrexate (5 mg/kg) and bradykinin (0.5 µM) was repeated. The number of leaky sites and clearance of FITC-dextran were determined during each intervention. The concentration of indomethacin used in these studies was based on previous studies in our laboratory and a report in the literature (11, 12, 25, 28).

Effects of methotrexate on adenosine-induced responses. The purpose of these studies was to determine the specificity of
methotrexate potentiation of bradykinin-induced responses by
determining its effects on adenosine-induced leaky site formation and increase in clearance of FITC-
dextran from the cheek pouch. Adenosine increases macromo-
ellecular efflux from the cheek pouch through a receptor-
mediated, NO-independent mechanism(s) (12, 15, 22, 39). After bicarbonate buffer was suffused for 30 min (equilibra-
tion period), FITC-dextran was injected intravenously, and the number of leaky sites and clearance of FITC-dextran were
determined for 30 min. Thereafter, adenosine (0.5 µM) was
suffused for 5 min before and after methotrexate (5 mg/kg)
was suffused for 30 min (15, 22, 39). The number of leaky sites and clearance of FITC-dextran were determined during each intervention. In preliminary studies, we determined that repeated suffusions of adenosine (0.5 µM) were associated with reproducible results. The concentration of adenosine
used in these studies was based on previous studies in our
laboratory and reports in the literature (11, 14, 15, 22, 39).

Effects of methotrexate on ionomycin-induced responses. The purpose of these studies was to determine whether
methotrexate potentiation of bradykinin-induced responses
through the l-arginine/NO biosynthetic pathway is mediated, in
part, by a receptor-dependent mechanism(s) by determin-
ing its effects on ionomycin-induced leaky site formation and
increase in clearance of FITC-dextran from the cheek pouch. Calcium ionophores, such as A-23187 and ionomycin, elicit endothelium-dependent, receptor-independent production of
NO or a related compound(s) in the cheek pouch (11, 19). After bicarbonate buffer was suffused for 30 min (equilibration period), FITC-dextran was injected intravenously, and the
number of leaky sites and clearance of FITC-dextran were
determined for 30 min. Then, two concentrations of ionomy-
cin (0.1 and 1.0 nM) were suffused for 5 min in an arbitrary
order. At least 45 min elapsed between subsequent suffusions of ionomycin (11, 19). Once suffusion of ionomycin was
stopped and the number of leaky sites returned to baseline,
methotrexate (5 mg/kg) was suffused for 30 min and suffusion of ionomycin (0.1 and 1.0 nM) was repeated. The number of
leaky sites and clearance of FITC-dextran were determined
during each intervention. In preliminary studies, we
determined that repeated suffusions of ionomycin (0.1 and 1.0
nM) were associated with reproducible results. The concentra-
tions of ionomycin used in these studies were based on a previous study in our laboratory and a report in the literature (11, 19).

Drugs. FITC-dextran, methotrexate, bradykinin, l-NAME,
d-NAME, l-arginine, d-arginine, ZnCl2, indomethacin, adenos-
ine, ionomycin, Escherichia coli nitrate reductase, NaNO2,
NaNO3, and 3-isobutyl-1-methylxanthine were obtained from
Sigma Chemical (St. Louis, MO). Indomethacin was dissolved
in sodium bicarbonate. All drugs were diluted in saline to the
desired concentrations on the day of the experiment.

Data and statistical analyses. When a test compound was
suffused over the cheek pouch, we determined the maximal
change in the number of leaky sites and clearance of FITC-
dextran and used it as the response to that compound. Data
are expressed as means ± SE. Because the number of leaky
sites and clearance of FITC-dextran returned to baseline
between successive suffusions of test compounds, all control
(saline) data are expressed as a single value for each experi-
mental condition. Statistical analysis was performed using
two-way analysis of variance and the Newman-Keuls test for
multiple comparisons. P < 0.05 was considered significant,
and n is given as the number of experiments, with each
experiment representing a separate animal.

RESULTS

Effects of Methotrexate on
Bradykinin-Induced Responses

Suffusion of methotrexate (5 mg/kg) alone for 30 min
was not associated with visible leaky site formation and
increase in clearance of FITC-dextran (data not shown; n = 6).
However, it significantly potentiated bradykinin-
induced responses (Fig. 1; each group, n = 6; P < 0.05).

Downloaded from http://ajpregu.physiology.org/ by 10.220.33.1 on July 9, 2017
The number of leaky sites increased significantly from 10 ± 1.01 cm² during suffusion of bradykinin (0.5 µM) alone to 20 ± 2.11 cm² during suffusion of methotrexate (5 mg/kg) and bradykinin (0.5 µM; Fig. 1A; each group, n = 6; P < 0.05). Likewise, clearance of FITC-dextran increased significantly from 1.2 ± 0.45 ml/min × 10⁻⁶ during suffusion of bradykinin (0.5 µM) alone to 2.6 ± 0.64 ml/min × 10⁻⁶ during suffusion of methotrexate (5 mg/kg) and bradykinin (0.5 µM; Fig. 1B; each group, n = 6; P < 0.05).

**Effects of L-NAME**

Suffusion of L-NAME, but not D-NAME (each, 100 µM), abrogated the potentiating effects of methotrexate (5 mg/kg)-induced responses (Fig. 2; each group, n = 4). The number of leaky sites increased significantly from 6 ± 1.01 cm² during suffusion of L-NAME (100 µM), methotrexate (5 mg/kg), and bradykinin (0.5 µM; Fig. 2A; each group, n = 4; P < 0.05). Clearance of FITC-dextran also increased significantly from 30.3 ± 4.6 ml/min × 10⁻⁶ during suffusion of L-NAME (100 µM), methotrexate (5 mg/kg), and bradykinin (0.5 µM; Fig. 2B; each group, n = 4; P < 0.05).

**Fig. 1.** Effects of suffusion of methotrexate (5 mg/kg; hatched bars) for 30 min on bradykinin (solid bars; 5 min)-induced leaky site formation (A) and increase in clearance of fluorescein isothiocyanate (FITC)-dextran (B) from the cheek pouch. Values are means ± SE; each group, n = 6 experiments. *P < 0.05 vs. control (saline; open bars). †P < 0.05 vs. bradykinin alone.

**Fig. 2.** Effects of suffusion of N[G-nitro-L-arginine methyl ester (L-NAME; 100 µM), N[G-nitro-D-arginine methyl ester (D-NAME; 100 µM), L-arginine (L-Arg; 1 mM) with L-NAME (100 µM), or D-arginine (D-Arg; 1 mM) with L-NAME (100 µM) for 30 min on methotrexate (MTX; 5 mg/kg)-induced potentiating effects of bradykinin (BK; 0.5 µM)-induced leaky site formation (A) and increase in clearance of FITC-dextran (B) from the cheek pouch. Values are means ± SE; each group, n = 4 experiments. *P < 0.05 vs. control (saline). †P < 0.05 vs. BK alone. ¶P < 0.05 vs. MTX and BK.
Table 1. Nitrites concentration and cGMP-like immunoreactivity in cheek pouch suffusate

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Nitrites, µM</th>
<th>cGMP, pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bicarbonate buffer</td>
<td>0.15 ± 0.05</td>
<td>ND</td>
</tr>
<tr>
<td>Control (saline)</td>
<td>0.93 ± 0.09</td>
<td>ND</td>
</tr>
<tr>
<td>Methotrexate (5 mg/kg)</td>
<td>7.06 ± 0.19*</td>
<td>58.95 ± 0.29*</td>
</tr>
<tr>
<td>Bradykinin (0.5 µM)</td>
<td>9.35 ± 0.63*</td>
<td>115.90 ± 2.50†</td>
</tr>
<tr>
<td>Methotrexate and bradykinin</td>
<td>12.00 ± 0.32†</td>
<td>201.45 ± 3.00‡</td>
</tr>
<tr>
<td>L-NAME (100 µM)</td>
<td>4.06 ± 0.40*</td>
<td>ND</td>
</tr>
<tr>
<td>L-NAME, methotrexate, and bradykinin</td>
<td>9.03 ± 0.27*</td>
<td>28.05 ± 0.25§</td>
</tr>
</tbody>
</table>

Values are means ± SE; each group, n = 4 experiments. ND, not detected; L-NAME, NÖ-nitro-L-arginine methyl ester. *P < 0.05 in comparison to control; †P < 0.05 in comparison to methotrexate; ‡P ≤ 0.05 in comparison to methotrexate and bradykinin alone; §P < 0.05 in comparison to methotrexate and bradykinin.

Table 1 reflects the nitrites concentration in the suffusate relative to suffusion of methotrexate and bradykinin alone (Table 1; 12.00 ± 0.32 vs. 7.06 ± 0.19 and 9.35 ± 0.63 µM, respectively; each group, n = 4; P < 0.05). L-NAME (100 µM) abrogated the increase in nitrites concentration in the suffusate during suffusion of methotrexate and bradykinin (Table 1).

Effects of ZnCl2

Suffusion of ZnCl2 (100 µM) had no significant effects on bradykinin (0.5 µM)-induced leaky site formation and increase in clearance of FITC-dextran (Fig. 3A; each group, n = 4; P > 0.5). ZnCl2 (100 µM) had also no significant effects on methotrexate (5 mg/kg) potentiation of bradykinin (0.5 µM)-induced responses (Fig. 3B; each group, n = 6; P > 0.5). The number of leaky sites was 24 ± 2/0.11 cm² during suffusion of methotrexate (5 mg/kg) and bradykinin (0.5 µM), and 21 ± 2/0.11 cm² during suffusion of ZnCl2 (100 µM), methotrexate (5 mg/kg), and bradykinin (0.5 µM; Fig. 3B, top; each group, n = 6; P > 0.5). Likewise, clearance of FITC-dextran was 32.6 ± 5.2 ml/min × 10⁻⁶ during suffusion...
of methotrexate (5 mg/kg) and bradykinin (0.5 µM), and 29.1 ± 3.7 ml/min \times 10^{-6} during suffusion of ZnCl₂ (100 µM), methotrexate (5 mg/kg), and bradykinin (0.5 µM; Fig. 3, bottom; each group, n = 6; P > 0.5).

Effects of Indomethacin

Indomethacin (10 mg/kg iv) had no significant effects on methotrexate (5 mg/kg) potentiation of bradykinin (0.5 µM)-induced responses (Fig. 4; each group, n = 7; P > 0.5). The number of leaky sites was 25 ± 3/0.11 cm² during suffusion of methotrexate (5 mg/kg) and bradykinin (0.5 µM) and was 22 ± 3/0.11 cm² after intravenous infusion of indomethacin (10 mg/kg) and suffusion of methotrexate (5 mg/kg) and bradykinin (0.5 µM; Fig. 4A; each group, n = 7; P > 0.5). Clearance of FITC-dextran was 40.1 ± 7.0 ml/min \times 10^{-6} during suffusion of methotrexate (5 mg/kg) and bradykinin (0.5 µM) and was 31.3 ± 7.0 ml/min \times 10^{-6} after intravenous infusion of indomethacin (10 mg/kg) and suffusion of methotrexate (5 mg/kg) and bradykinin (0.5 µM; Fig. 4A; each group, n = 7, P > 0.5).

Effects of Methotrexate on Adenosine-Induced Responses

Suffusion of methotrexate (5 mg/kg) had no significant effects on adenosine (0.5 µM)-induced leaky site formation and increase in clearance of FITC-dextran (Fig. 5; each group, n = 7; P > 0.5). The number of leaky sites was 10 ± 1/0.11 cm² during suffusion of adenosine (0.5 µM) alone, and 9 ± 2/0.11 cm² during suffusion of methotrexate (5 mg/kg) and adenosine (0.5 µM; Fig. 5A; each group, n = 7; P > 0.5). Clearance of FITC-dextran was 28.3 ± 3.6 ml/min \times 10^{-6} during suffusion of adenosine (0.5 µM) alone, and 25.8 ± 5.6 ml/min \times 10^{-6} during suffusion of methotrexate (5 mg/kg) and adenosine (0.5 µM; Fig. 5B; n = 7; P > 0.5).
Effects of Methotrexate on Ionomycin-Induced Responses

Suffusion of methotrexate (5 mg/kg) had no significant effects on ionomycin (0.1 and 1.0 nM)-induced responses (Fig. 6; each group, n = 6; P > 0.5). The number of leaky sites was 9 ± 2/0.11 cm² during suffusion of ionomycin (1.0 nM) alone and was 6 ± 1/0.11 cm² during suffusion of methotrexate (5 mg/kg) and ionomycin (1.0 nM; Fig. 6A; each group, n = 6; P > 0.5). Clearance of FITC-dextran was 28.6 ± 6.7 ml/min × 10⁻⁶ during suffusion of ionomycin (1.0 nM) alone, and 30.3 ± 6.0 ml/min × 10⁻⁶ during suffusion of methotrexate (5 mg/kg) and ionomycin (1.0 nM; Fig. 6B; each group, n = 6; P > 0.5).

DISCUSSION

The results of this study show that methotrexate, at a concentration used in humans (3, 17), significantly potentiates bradykinin-induced increase in macromolecular efflux from the in situ hamster cheek pouch. These effects were mediated by the L-arginine/NO biosynthetic pathway because they were abrogated by L-NAME, but not D-NAME, and because L-arginine, but not D-arginine, abolished L-NAME-induced responses. Moreover, suffusion of methotrexate and bradykinin was associated with significant increases in nitrites concentration and cGMP-like immunoreactivity in the suffusate that were abrogated by L-NAME. The effects of methotrexate were not related to nonspecific damage to the endothelium because the number of leaky sites and clearance of FITC-dextran returned to baseline once suffusion of methotrexate and bradykinin was stopped.

We also found that methotrexate potentiation of bradykinin-induced responses were specific and receptor dependent because methotrexate had no significant effects on adenosine- and ionomycin-induced increases in macromolecular efflux, and because ZnCl₂ and indomethacin, at a concentration known to inhibit cyclooxygenase in the cheek pouch (25, 28), had no significant effects on methotrexate-induced responses. On balance, these data suggest that methotrexate potentiation of bradykinin-induced increase in macromolecular efflux from the in situ cheek pouch is mediated by a specific, receptor- and L-arginine/NO biosynthetic pathway-dependent mechanism(s) (19, 27, 29). Further studies are indicated to elucidate the cellular source(s) of NO or a related compound(s) produced in the cheek pouch during suffusion of methotrexate and bradykinin.

The hamster cheek pouch is an established model to investigate mechanisms underlying plasma exudation from the oral mucosa during the host inflammatory response to injury (11–15, 19, 20, 22, 25, 28, 30, 34, 35, 39). To this end, we and others showed that bradykinin is produced in the inflamed oral mucosa and increases macromolecular efflux from postcapillary venules, in part, through the L-arginine/NO biosynthetic pathway (1, 6, 13, 19, 20, 22, 23, 34; and D. R. Springall and I. Rubinstein, unpublished observations). Moreover, Gao et al. (11, 12) showed recently that the edemagenic effects of bradykinin in this organ are potentiated by potent endogenous phlogistic mediators, such as interleukin 1β and a stable analogue of vasoactive intestinal peptide, which by themselves have no significant effects on macromolecular efflux. These potentiating effects were mediated, in part, by the L-arginine/NO biosynthetic pathway. The results of this study support and extend these observations by showing that the L-arginine/NO biosynthetic pathway mediates methotrexate potentiation of bradykinin-induced increase in macromolecular efflux from the in situ cheek pouch. Whether other antineoplastic drugs amplify the edemagenic effects of bradykinin in the oral mucosa by similar mechanisms remains to be determined (7, 10, 32, 33, 37).

Rubinstein and Mayhan (27) showed that suffusion of L-NAME onto the cheek pouch is associated with significant vasoconstriction. Hence, changes in vasoconstrictor tone and/or venular driving pressure in the cheek pouch might have mediated, in part, methotrexate...
potentiation of bradykinin-induced responses and the attenuating effects of L-NAME. However, this possibility seems unlikely because methotrexate had no significant effects on adenosine- and ionomycin-induced increases in macromolecular efflux. In addition, Tomeo and Durán (35) showed that platelet-activating factor increases macromolecular efflux from the cheek pouch while at the same time eliciting potent vasoconstriction. Finally, Murray et al. (22) showed that bradykinin-induced increase in macromolecular efflux from this organ is not mediated by changes in venular driving pressure. Overall, these data suggest that the effects of methotrexate and L-NAME on bradykinin-induced increase in macromolecular efflux observed in this study could not be attributed to local changes in vasomotor tone or venular driving pressure.

Although suffusion of methotrexate alone was associated with a significant increase in nitrites concentration and cGMP-like immunoreactivity in the suffusate, which was of smaller magnitude relative to that associated with suffusion of bradykinin, it nonetheless had no significant effects on macromolecular efflux. These data are consistent with previous studies in hamsters and rats showing that acute administration of methotrexate is not associated with oral mucositis (10, 30, 32). However, methotrexate potentiated bradykinin-induced increase in macromolecular efflux, and this response was associated with significant increases in nitrites concentration and cGMP-like immunoreactivity in the suffusate, which were greater than those associated with suffusion of each of these compounds alone. The smaller increment in cGMP-like immunoreactivity in the absence of significant changes in macromolecular efflux during suffusion of methotrexate alone suggests that disruption of the barrier function of postcapillary venules in the cheek pouch could be partly related to the magnitude of local cGMP production. Alternatively, nitrites and cGMP-like immunoreactivity produced in the cheek pouch during suffusion of methotrexate alone might be derived from cells not directly involved in regulation of barrier function of postcapillary venules. Clearly, additional studies are warranted to support or refute these hypotheses.

Previous studies showed that inactivation of alcohol dehydrogenase by NO and peroxynitrite is partly related to chelation of the zinc moiety in the catalytic domain of the enzyme (5, 16). In addition, Park and Means (24) showed that sodium nitroprusside, an NO donor, inactivates angiotein-converting enzyme inhibitor (ACE), a zinc metalloenzyme that hydrolyzes bradykinin very effectively in the cheek pouch (12, 26, 39). Conceivably, methotrexate potentiation of bradykinin-induced increase in macromolecular efflux from this organ could have been related, in part, to chelation of zinc moiety in the catalytic domains of ACE or other metalloenzymes that hydrolyze bradykinin thereby slowing local peptide catabolism and amplifying macromolecular efflux (1, 24, 26, 36, 39). Although we cannot refute this possibility, it nonetheless seems unlikely because suffusion of a relatively high concentration of ZnCl₂ to allow reincorporation of Zn²⁺ had no significant effects on methotrexate potentiation of bradykinin-induced responses.

Perspectives

The specific interaction between methotrexate and bradykinin to amplify macromolecular efflux from the oral mucosa observed in this study suggests that premorbid phenotypic expression of phlogistic mediators in the oral mucosa could play a role in the genesis of oral mucositis during methotrexate therapy.

In summary, we found that methotrexate amplifies bradykinin-induced increase in macromolecular efflux from the in situ oral mucosa in a specific, receptor- and L-arginine/NO biosynthetic pathway-dependent fashion.

This study was supported, in part, by grants from the National Institutes of Health (DE-10347), American Heart Association of Metropolitan Chicago, and Laerdal Foundation for Acute Medicine.

I. Rubinstein is a recipient of a Research Career Development Award from the National Institutes of Health (DE-00036) and a University of Illinois Scholar Award.

Address for reprint requests: I. Rubinstein, Dept. of Medicine (M/C 787), Univ. of Illinois at Chicago, 840 South Wood St., Chicago, IL 60612-7323.

Received 13 November 1996; accepted in final form 23 May 1997.

REFERENCES


