**γ-Aminobutyric Acid Is Synthesized and Released by the Endothelium**

**Potential Implications**

Suvajit Sen, Sohini Roy, Gautam Bandyopadhyay, Bari Scott, Daliao Xiao, Sivakumar Ramadoss, Sushil K. Mahata, Gautam Chaudhuri

**Rationale:** Gamma aminobutyric acid (GABA), a neurotransmitter of the central nervous system, is found in the systemic circulation of humans at a concentration between 0.5 and 3 μmol/L. However, the potential source of circulating GABA and its significance on the vascular system remains unknown. We hypothesized that endothelial cells (ECs) may synthesize and release GABA to modulate some functions in the EC and after its release into the circulation.

**Objective:** To assess whether GABA is synthesized and released by the EC and its potential functions.

**Methods and Results:** Utilizing the human umbilical vein ECs and aortic ECs, we demonstrated for the first time that ECs synthesize and release GABA from [1-14C]glutamate. Localization of GABA and the presence of the GABA-synthesizing enzyme, glutamic acid decarboxylase in EC were confirmed by immunostaining and immunoblot analysis, respectively. The presence of GABA was further confirmed by immunochemistry in the EC lining the human coronary vessel. EC-derived GABA regulated the key mechanisms of ATP synthesis, fatty acid, and pyruvate oxidation in EC. GABA protected EC by inhibiting the reactive oxygen species generation and prevented monocyte adhesion by attenuating vascular cell adhesion molecule -1 and monocyte chemoattractant protein-1 expressions. GABA had no relaxing effect on rat aortic rings. GABA exhibited a dose-dependent fall in blood pressure. However, the fall in BP was abolished after pretreatment with pentolinium.

**Conclusions:** Our findings indicate novel potential functions of endothelium-derived GABA. (Circ Res. 2016;119:621-634. DOI: 10.1161/CIRCRESAHA.116.308645.)

**Key Words:** adenosine triphosphate ■ endothelial cell ■ fatty acids ■ gamma-aminobutyric acid ■ pyruvate

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G gamma aminobutyric acid (GABA) was first identified as a biochemical substance in 1910 by Ackermann and Kutscher who showed that putrefactive bacteria could produce it by decarboxylation of glutamic acid. It was later found in many microorganisms and plant tissues and in 1950; its presence in considerable amounts in mammalian brain was also reported. Subsequently, it was detected in the peripheral nervous system, the β cells of the pancreas and various other tissues including the gastrointestinal tract and the oviduct.

Several important circulating vasoactive substances like prostacyclin and nitric oxide synthesized by the endothelium are released into the circulation where they have significant physiological actions. A decrease in the synthesis and release of these vasoactive substances are associated with pathophysiological conditions. GABA is present in significant quantities in the plasma of humans. Intermittent synthesis and release of GABA by the β cells of the pancreas and that formed by the gut microbiota may not fully account for the circulating levels of GABA (0.5–3 μmol/L) detected in humans. Therefore, we decided to assess whether GABA was synthesized and released by the endothelial cells similar to other vasoactive substances and if so, its potential physiological actions in the endothelial cells and in the circulation.

**Methods**

**Materials**

1- Allylglycine (LAG), catalog A7762, and GABA, catalog A2129, were obtained from Sigma-Aldrich Corp., MO. Gabaculine (Gc) was obtained from Santa Cruz Biotechnology Inc, TX, catalog sc-200473. [1-14C]Pyruvate, [1-14C]glutamate and [8-14C]ADP were obtained from Santa Cruz Biotechnology Inc, TX, catalog sc-200473.

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This work was also partially presented at the Deuel Conference, March 2015, to honor Michael Brown and Joseph Goldstein.

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Nonstandard Abbreviation and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>FFA</td>
<td>free fatty acid</td>
</tr>
<tr>
<td>FFA0</td>
<td>FFA oxidation</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma aminobutyric acid</td>
</tr>
<tr>
<td>GAD</td>
<td>glutamic acid decarboxylase</td>
</tr>
<tr>
<td>HAEC</td>
<td>human aortic endothelial cells</td>
</tr>
<tr>
<td>HSF</td>
<td>human skin fibroblast</td>
</tr>
<tr>
<td>HUVECs</td>
<td>human umbilical vein endothelial cells</td>
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<tr>
<td>LAG</td>
<td>l-allylglycine</td>
</tr>
<tr>
<td>MCP</td>
<td>monocyte chemotactic protein</td>
</tr>
<tr>
<td>Scr</td>
<td>scrambled control</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>VCAM</td>
<td>vascular cell adhesion molecule</td>
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from Perkin Elmer, MA; catalog NEC290E050UC, NEC25S050UC, and NEC559010UC. All other chemicals unless specified were obtained from Sigma.

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were freshly isolated from umbilical cords from different donors after normal delivery (with approval from the Institutional Review Board of University of California at Los Angeles, LA, CA). Primary cultures of HUVEC were prepared as described and used at passages 1 or 2. Primary cultures of human aortic endothelial cells (HAEC) at passage 2 or 3 were a generous gift from Dr M. Navab in the Department of Medicine, University of California, Los Angeles. HSF and THP1 were cultured in DMEM supplemented with 5% FCS and dissolved in 50-μL detergent (1% NP40)-containing lysis buffer by pipetting up and down. An aliquot was assayed for protein, and 5 μL of both cell lysate and medium was spotted on a TLC plate along with reference unlabeled glutamic acid and GABA. The plate was run twice in the same developing solvent and in between the 2 runs, the plate was air dried completely. The developing solvent was n-butanol/acetic acid/water: 66:10:20. After the run and drying, spots were visualized by ninhydrin spray and exposure of the plate at 60°C for 10 minutes. Thereafter, glutamic acid and GABA spots were marked and scapped off into vials for radioactive counting. Recovery of radioactivity on the TLC was 84%, and counting efficiency was 90%. The results represent the average of 6 independent experiments.


HUVECs were cultured in 96-well plates. On reaching 70% to 80% confluence, medium was changed to HEPES-Krebs-Ringer-bicarbonate buffer containing 0.2% BSA and 0.4 mmol/L glutamic acid. An aliquot of [1-14C]glutamic acid (0.8 μCi) was added to each well. Final volume was 50 μL/well. The plate was incubated for 2 hours in the 5% CO2 incubator. Cultured media were then collected, and cells were washed and dissolved in 50-μL detergent (1% NP40)-containing lysis buffer by pipetting up and down. An aliquot was assayed for protein, and 5 μL of both cell lysate and medium was spotted on a TLC plate along with reference unlabeled glutamic acid and GABA. The plate was run twice in the same developing solvent and in between the 2 runs, the plate was air dried completely. The developing solvent was n-butanol/acetic acid/water: 66:10:20. After the run and drying, spots were visualized by ninhydrin spray and exposure of the plate at 60°C for 10 minutes. Thereafter, glutamic acid and GABA spots were marked and scapped off into vials for radioactive counting. Recovery of radioactivity on the TLC was 84%, and counting efficiency was 90%. The results represent the average of 6 independent experiments.

Detection of GABA by ELISA

GABA was detected using an ELISA-based kit from LDN Labor Diagnostika, Nord GmbH & Company KG, Nordhorn, Germany, catalog BA E-2500 as described by others. Briefly, the immunosay was performed after extraction and derivatization of GABA from experimental samples. The antigen was bound to the solid phase of the microtitre plate. The acylated analyte concentrations and the solid phase bound analyte competed for a fixed number of antiserum binding sites. When the system was in equilibrium, free antigen and free antigen–antibody complexes were removed by washing. The antibody bound to the solid phase was detected by an anti-rabbit IgG-peroxidase conjugate using TMB as a substrate. The primary antibody was raised against BSA-conjugated GABA. The reaction was monitored at 450 nm. Quantification of unknown samples is achieved by comparing their absorbance with a standard curve prepared with known standards.

HUVEC and HAEC homogenates were incubated with pyridoxal phosphate and glutamate as discussed earlier in a total reaction volume of 300 μL. The reaction was stopped by the addition of equal volume of chloroform and PBS and centrifuged as described earlier. The aqueous layer was then extracted and heated at 37°C for 30 minutes to get rid of traces of chloroform. It was then applied in the ELISA plates for the detection of GABA. The amount of GABA was analyzed against a standard curve generated by known GABA concentrations as detailed in the product manual.

GABA released from cells was estimated from the spent media. HUVEC was subjected to treatments such as LAG and Gc, which are specific inhibitors of GAD and GABA transaminase, respectively. LAG was used at 300 μmol/L and Gc at 45 μmol/L concentrations for 24 hours. The concentrations utilized were based on concentrations that yielded optimum effects in predetermined trails (Online Figures I and II) and from those used by others. The amount of GABA was corrected against the read-out of media without cells and then further against the total protein in the cells. The results represent the average of 6 independent experiments.

Detection of GABA in HUVEC and in Blood Vessels by Immunostaining

HUVEC were grown in 35 mmol/L glass bottom cell culture dishes obtained from MatTek corporation, Ashland, MA, catalog P35G-0.170-14-C. Cells were grown in endothelial cell basal medium, until 60%
confluent. Thereafter, cells were fixed in 4% paraformaldehyde at room temperature for 10 minutes and then permeabilized with 0.1% Triton X-100 for 5 minutes at room temperature. Permeabilized cells were rinsed 3× with PBS and incubated in blocking solution (1% bovine serum albumin/phosphate buffer saline with 0.1% Tween 20 [PBST]) for 30 minutes at room temperature to remove nonspecific binding of the antibody. Cells were rinsed 3× in PBS between each of the steps. GABA was detected using mouse monoclonal anti-GABA antibody in a 1:500 dilutions (overnight treatment at 4°C), obtained from Abcam (catalog 86186), Cambridge, MA, as per manufacturer’s instruction. All subsequent steps were performed at room temperature. Fluorescein isothiocyanate–conjugated goat anti-mouse secondary antibody (catalog 10006617; Cayman Chemicals, MI) was utilized in 1:10000 dilutions, for fluorescence detection of signals. Nonspecific rabbit IgG (catalog sc-2027; Santa Cruz Biotechnology Inc, TX) and fluorescein isothiocyanate–conjugated goat anti-rabbit IgG (catalog 10006588; Cayman Chemicals) were used for control staining. Cells were counterstained for nuclei with Hoechst 33342. Results are representative of at least 3 experiments.

Sections of normal adult human heart tissue were obtained from Translational Pathology Core Laboratory, UCLA. Selected slides were stained with von Willebrand factor at 1:200 dilutions to detect the endothelial cells in the blood vessels of coronary circulation. GAD65/67 (EMD Millipore cat AB1511) and GABA (Abcam cat 86186) was detected in consecutive sections, utilizing rabbit polyclonal anti-GAD65/67 antibody and mouse monoclonal anti-GABA antibody, respectively, in 1:500 dilutions as per manufacturer’s instruction.

Detection of Steady State Levels of ATP From HUVEC
HUVEC either untreated or treated with Gc and LAG separately as well as GDAsh HUVEC and Scr HUVEC, were suspended in PBS, lysed with chloroform, and ATP was detected utilizing luciferase/luciferin-coupled reactions as described earlier. An equal volume of cell suspension was utilized for protein estimation. Data are representative of at least 6 individual experiments.

Detection of GABA-Induced Synthesis of [1-14C] ATP Synthesis From [8-14C]ADP
HUVEC were scraped off the plates in 1-mL buffer-1 (100 mmol/L KCl, 50 mmol/L Tris-HCl, 1 mmol/L EDTA, 5 mmol/L MgCl2, 2 mmol/L ATP, pH 7.4) and homogenized by 10 strokes in an all-glass Dounce homogenizer. The homogenate (containing intact mitochondria) was centrifuged at 800g for 10 minutes, to separate the nuclei and cell debris. Fifty microliters of the supernatant was set aside for protein estimation. Supernatants (100–200 μg) were incubated with [1-14C]palmitate along with various concentrations of GABA (0–10 μmol/L).

To assess 14CO2 production, incubation was performed inside 4-mL glass vials containing a conical glass insert (300 μL in volume). Inserts carry 0.2–0.5 mL NaOH or an organic solvent mixture (ethanolamine/ethylen glycol; 1:2). The vials were sealed with a rubber stopper. To the 40-μL homogenates (100- to 200-μg protein), 160-μL aliquots of reaction mixtures containing (final concentration) 100 mmol/L sucrose, 10 mmol/L Tris-HCl, 5 mmol/L KH2PO4, 80 mmol/L MgCl2, 2 mmol/L L-carnitine, 0.1 mmol/L/ L malate, 2 mmol/L ATP, 0.05 mmol/L CoA, 1 mmol/L DTT, 0.2 mmol/L EDTA, 0.3% BSA, pH 7.4, and 0.1 μCi [1-14C]palmitate (0.5 μCi/mL; 0.2 mmol/L) were added. Palmitate was delivered as an aqueous suspension complexed with fatty acid free BSA (palmitate/BSA=2.5:1). After 1-hour incubation at 37°C, a 100-μL aliquot of 70% perchloric acid was injected into each vial, outside the conical insert. The vials were incubated for 2 hours at 4°C or kept overnight in the cold room

Subsequently, the contents of the inserts were transferred to scintillation vials for counting 14CO2 radioactivity directly if organic solvent was used or after neutralization with 0.2-μL 2 N NaOH was used. The acidified reaction mixture from each incubation vial was transferred to a microfuge tube, neutralized with 100-μL 3 N KOH and centrifuged at 10000g for 10 minutes. Pellets were saved. The supernatants were transferred to scintillation vials for counting radioactivity. This fraction of radioactivity in the acidified (by perchloric acid) aqueous reaction mixture represents acid soluble metabolites (ASM) of [1-14C]palmitate. The 2 radioactive samples (ASM+14CO2) were combined to represent total palmitate oxidation.

To determine mitochondrial uptake, the HUVEC homogenate as described above was incubated with GABA and after 1-hour incubation at 37°C in buffer-1. After this, the incubation mixture was centrifuged at 8000g to obtain mitochondrial pellets. The mitochondrial pellet was washed twice and counted for radioactivity to assess mitochondria-bound radioactivity (as relevant for mitochondrial uptake of palmitate). Figures represent data averaged from 6 experiments.

Detection of [1-14C]Pyruvate Oxidation
The detection of [1-14C]pyruvate oxidation is based on the principle that pyruvate is also converted to acetyl CoA in the mitochondria which is then incorporated into the TCA cycle and 14CO2 is formed as the end product. Therefore, determination of the amount of 14CO2 indicates the amount of [1-14C]palmitate oxidation. [1-14C]palmitate uptake in the mitochondria and subsequent detection of 14CO2 oxidation was performed as described others with minor modifications. HUVEC were scraped off 10-cm plates in 1 mL of buffer-1 (100 mmol/L KCl, 50 mmol/L Tris-HCl, 1 mmol/L EDTA, 5 mmol/L MgCl2, 1 mmol/L ATP, pH 7.4) and homogenized by 10 strokes in an all-glass Dounce homogenizer. The homogenate (containing intact mitochondria) was centrifuged at 800g for 10 minutes, to separate the nuclei and cell debris. Fifty microliters of the supernatant was set aside for protein estimation. Supernatants (100–200 μg) were incubated with [1-14C]palmitate along with various concentrations of GABA (0–10 μmol/L).

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Detection of Monocyte Adhesion
Monocyte adhesion assays were performed utilizing a commercial kit from Millipore, Billerica, MA, catalog ECM645, as described before. HUVEC were grown to 80% confluence in specific petridishes and treated with drugs as indicated in the results under flow conditions. Thereafter cells were washed twice with fresh media and incubated with monocytes (THP1) treated with 2.5 μmol/L Calcein AM for 1 hour. Cells were then washed with fresh media before measuring fluorescence at excitation and emission...
wavelengths of 488 and 530 nm, respectively. Results were averaged from 12 wells for each treatment. Thereafter, percent changes were calculated and represented as indicated in results. Experiments repeated under a different method using a metabolic shaker\textsuperscript{30} yielded similar results (data not shown).

**Detection of Intracellular Reactive Oxygen Species**

H\textsubscript{2}O\textsubscript{2} production was determined using the Amplex Red hydrogen peroxide assay kit (Invitrogen, A22188) as per the manufacturer’s protocol and as described before.\textsuperscript{30} Briefly, 80,000 cells were added to 100 μL of an enzyme assay buffer (50 mmol/L potassium phosphate buffer, pH 7.4) containing 0.1 U/mL of horseradish peroxidase and 50 μmol/L Amplex Red. The conversion of Amplex Red to resorufin (Ex/Em: 570/585 nm) was measured over time. The maximum fluorescence intensity was achieved at 4 hours after exposure to the Amplex Red reagent. Pegylated Catalase (0.1 U/mL; PEG-CAT) was utilized as a negative control.

**Detection of mRNA Levels of Monocyte Chemoattractant Protein 1 and Vascular Cell Adhesion Molecule 1**

RNA was isolated from untreated and LAG-treated HUVECs with TRIzol Reagent, and cDNA was prepared with iScript Reverse Transcription Supermix (cat 170–8841; BIO-RAD, CA). Quantitative polymerase chain reaction was performed in a BIO-RAD C1000 Thermal Cycler to estimate the fold-change in mRNA expression of monocyte chemoattractant protein (MCP) 1 and vascular cell adhesion molecule (VCAM) 1 using the iQaq Universal SYBR Green Supermix (cat 72–5124; BIO-RAD). The MCP1, VCAM1, and GAPDH primers were synthesized by Life Technologies Corporation, New York. The sizes of amplics were 141 bp (human VCAM1: CCGATGGTCGTCTGAGATTGGA as forward primer, AGTGAATGGTGTCCTCCA as reverse primer), 137 bp (human MCP1: TGCAGAGGCTCGCGAGCTA as forward primer, CAGTTGGTCTGAGATTGGA as reverse primer), and 225 bp (human GAPDH: GCCAAAGGTTGCTATCTC as forward primer, GGCCATCCAAGCTTCT as reverse primer).

**Immunoblot Analysis**

HAEC and HUVEC were harvested on reaching 80% confluency, and the pellets were lysed with NP-40 buffer in the presence of Protease Inhibitor Cocktail (catalog P-8340 Sigma-Aldrich Corp., MO). The samples were run on a 10% Bis-Tris gel and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% non-fat milk in 1×TBS-Tween to remove nonspecific antibody binding. Samples were run on a 10% Bis-Tris gel and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% non-fat milk in 1×TBS-Tween to remove nonspecific antibody binding. Anti-GAD 65 and 67 Rabbit Polyclonal Antibody (catalog AB1511; Santa Cruz Biotechnology Inc), 1:500 dilution was used as primary and goat antimouse IgG-horseradish peroxidase (catalog sc-2005; Santa Cruz Biotechnology Inc), 1:5000 dilution as secondary antibody. The sizes of amplics were 141 bp (human MCP1: TGCAGAGGCTCGCGAGCTA as forward primer, CAGTTGGTCTGAGATTGGA as reverse primer), 137 bp (human GAPDH: GCCAAAGGTTGCTATCTC as forward primer, GGCCATCCAAGCTTCT as reverse primer).

**Measurement of Blood Pressure in Rats**

Experiments were conducted in 3-month-old male Sprague-Dawley rats (+400–500 g). Rats were anesthetized with inhalation of isoflurane (5% for induction, 2% for maintenance). Polyethylene catheters (PE50) were inserted into the left femoral artery and the left femoral vein for arterial BP recording and drug infusion, respectively. BP was recorded continuously throughout each study with data acquisition software (Powerlab 16/SP and chart version 5; AD Instruments), as described previously.\textsuperscript{30} Body temperature was measured with a rectal thermometer probe and maintained at 37°C with a heating pad. BP responses were initially tested by bolus intravenous injections of phenylephrine (20 μg/kg) that increased BP, acetylcholine (5 μg/kg), and sodium nitroprusside (0.3 μmol/kg) that decreased BP. After BP recovery, animals received intravenous GABA (0.1, 0.3, and 1.0 mg/kg), and the dose–responses of GABA were repeated after intravenous injection of L-NAME (15 mg/kg). To determine a central effect of GABA, the dose–responses of GABA on BP were repeated after intravenous injection the ganglion blocker pentolinium (5 mg/kg). At the end of GABA dosing, BP responses were tested again with phenylephrine (20 μg/kg) that increased BP and sodium nitroprusside (0.3 μmol/kg) that decreased BP. All animal experiments were approved by the Internal Review Board at University of Loma Linda (IACUC8140052).

**Statistical Analysis**

Data are presented as mean±SD. Student t test was performed for comparison between 2 groups. All paired t tests were calculated using the software Graphpad Prism 5. *P<0.05, **P<0.01, ***P<0.001 were considered statistically significant.

**Results**

**HAEC and HUVEC Homogenates Exhibited GAD Activity**

HAEC and HUVEC homogenates synthesized [1-\textsuperscript{14}C]GABA when incubated with [1-\textsuperscript{14}C]glutamate in the presence of pyridoxal phosphate. We observed ±20% and ±30% radiolabel incorporation in GABA from radiolabeled glutamate when incubated with homogenates from HAEC and HUVEC, respectively (Figure 1A). Synthesized GABA was separated from glutamate utilizing ion-exchange chromatography. The flow through (FT) and elution profiles of GABA and glutamate were verified using pure [1-\textsuperscript{14}C]GABA and [1-\textsuperscript{14}C]glutamate (Figure 1A, inset, left). The separation of GABA from glutamate by ion-exchange chromatography was further confirmed by TLC utilizing pure labeled GABA and glutamate as standards (Figure 1A, inset, right panel). Immunoblot analysis utilizing monoclonal antibodies against the human GAD65 and GAD67 confirmed their expression in both HAEC and HUVEC homogenates (Figure 1B).

GAD activity in homogenates derived from HAEC and HUVEC was further verified by an ELISA-based immune assay that indicated progressively increased synthesis of GABA on incubation with increasing concentrations of glutamate. GABA synthesis ranged from 0.8 to 2 μg and 0.8 to 3 μg utilizing homogenates from HAEC and HUVEC, respectively, after exposure to 0 to 50 mmol/L glutamate (Figure 2A and 2B). The optimum concentration of 0.250-μg homogenate protein, utilized for the above assays, was determined by a previous analysis of GABA production against increasing concentrations of protein from HUVEC homogenates. On
confirmation that both HUVEC and HAEC exhibited GAD activity, we proceeded to investigate whether endothelial cells in standard cultures produced GABA, under steady state conditions. We utilized HUVEC for further investigations.

**Cultured HUVEC Synthesized and Released GABA**

The most definitive proof of GABA synthesis, under steady state conditions, in a tissue type, is its capacity to synthesize radiolabeled GABA from its radiolabeled precursor, glutamate. We observed that HUVEC exposed to [1-14C]glutamate synthesized and released [1-14C]GABA in standard culture conditions. The percentage of total radioactivity incorporated in [1-14C]GABA, in the intracellular and extracellular compartments, were ≈40% and ≈30%, respectively, when exposed to [1-14C]glutamate added along with 0.4 mmol/L cold glutamate as present in standard culture conditions (Figure 3A and 3B). This phenomenon was verified by the ELISA-based assay. We observed that cultured HUVEC released ≈9 μg GABA/mg protein. There was a further increase by ≈18% in GABA levels when cells were pretreated with glutamate synthesized and released [1-14C]GABA in standard culture conditions. The percentage of total radioactivity incorporated in [1-14C]GABA, in the intracellular and extracellular compartments, were ≈40% and ≈30%, respectively, when exposed to [1-14C]glutamate added along with 0.4 mmol/L cold glutamate as present in standard culture conditions (Figure 3A and 3B). This phenomenon was verified by the ELISA-based assay. We observed that cultured HUVEC released ≈9 μg GABA/mg protein. There was a further increase by ≈18% in GABA levels when cells were pretreated with...
with Gc, an inhibitor of GABA transaminase that thereby enhances the endogenous half-life of GABA by preventing its inactivation. Conversely, the levels of released GABA decreased by ≈52.6% from untreated controls (Figure 3C), after exposure of the cells to LAG, a specific inhibitor of GABA synthesis. In addition, immunostaining analysis utilizing monoclonal antibodies against GABA confirmed its presence in HUVEC under steady-state conditions (Figure 3D).
and in the endothelial cells lining of coronary blood vessels as shown in cross section of heart tissue (Figure 3E, right panel). Endothelium lining of the coronary vessels was determined by the detection of von Willebrand factor (Figure 3E, left). Coronary vessel sections also revealed the presence of GAD65/67 (Online Figure III). Immunoblot analysis confirmed successful silencing of both GAD isoforms (Figure 3F, left) and an associated decrease in GABA release by ≈52% (Figure 3F). This was comparable to that observed with LAG treatment. We further compared GABA released from endothelial cells with those from PC12 cells derived from peripheral nervous tissues (pheochromocytoma of the rat adrenal medulla) and with those from other tissue types, such as a monocytes (THP1) and HSF. Comparison of the GAD65/67 protein expression levels, among the different cell types indicated that PC12 cells exhibited maximum expression, whereas HSF cells exhibited the least (Figure 3G, left). GAD65/67 expressed by HUVEC and HAEC were less than that of PC12 cells but significantly more than that of HSF cells (Figure 3G, left). A similar trend was observed in the levels of GABA released by the different cell types (Figure 3G, right). The GAD65/67 expression in THP1 cells was comparable to those in HAEC and HUVEC. However, GABA released by THP1 cells was significantly less than that of endothelial cells. This could be attributed to differential enzyme activities between the cell types.

GABA Maintained Homeostatic Levels of ATP in HUVEC

We next elucidated the role of GABA in the maintenance of cellular energetics in HUVEC by investigating its effect on the homeostatic levels of intracellular ATP. We observed that the steady-state levels of endogenous GABA were directly associated with ATP levels. HUVEC pretreated with Gc exhibited a ≈29% increase in cellular ATP levels. Conversely, pretreatment with LAG decreased ATP levels by ≈32% when compared with untreated controls (Figure 4A, left). Similar to that observed with pharmacological inhibition, silencing of GAD isoforms exhibited comparable reduction in ATP production (Figure 4A, middle). It was also observed that blocking of both GABAA and GABAB receptors, utilizing classical inhibitors such as picrotoxin and CGP55845 hydrochloride, respectively, inhibited endogenous ATP production in HUVEC (Figure 4A, right). This could be because of a mitigation of exogenous action of released GABA that will be further investigated in future studies. However, Gc rescued the decrease in ATP production in the presence of receptor blockers. This further confirmed that endogenous GABA significantly contributed toward the maintenance of steady-state intracellular ATP levels. We also investigated the formation of [1-14C]ATP from [8-14C]ADP, in HUVEC homogenates after exposure to increasing concentrations of GABA. We observed that a range of 0 to 4 μmol/L GABA elicited a maximum increase of ≈16% in ATP synthesis (Figure 4B).

GABA Promoted Pyruvate Oxidation in HUVEC Homogenates With Intact Mitochondria

We next investigated the mechanism(s) by which GABA induced ATP formation in HUVEC. We assessed whether GABA increased the oxidation of 2 critical components of cellular metabolism. First, we tested whether GABA increased the oxidation of pyruvate, which is one of the key metabolites that fuel the TCA cycle and hence an important determinant of homeostatic ATP levels. We observed that GABA in a concentration-dependent manner increased [14C]CO2 production from [1-14C]pyruvate in HUVEC homogenates with intact mitochondria. [14C]CO2 increased to a maximum of ≈20% over a concentration range of 0 to 10 μmol/L GABA (Figure 5A). GABA-mediated oxidation of pyruvate was observed even in the presence of Gc, confirming that GABA per se (and not a

Figure 4. A, Left, Estimation of steady state levels of ATP (utilizing the luciferin/luciferase system), in human umbilical vein endothelial cell (HUVEC), in the basal state or after exposure to gabaculine (Gc) that inhibits gamma aminobutyric acid (GABA) transaminase or L-Allylglycine (LAG) that inhibits GABA synthesis for 24 h in standard culture conditions. Data are presented as mean±SD. A, Middle, Estimation of steady state levels of ATP (utilizing the luciferin/luciferase system), in scrambled control (Scr) and GADsh human umbilical vein endothelial cells (HUVEC). A, Right, Estimation of steady-state levels of ATP (utilizing the luciferin/luciferase system) in untreated controls and Gc-treated cells in the presence and absence of receptor blockers (RB); picrotoxin for GABAA receptors and CGP55845 hydrochloride for GABAB receptors. Data are presented as mean±SD. B, Synthesis of [1-14C]-ATP from [8-14C]-ADP by homogenates of HUVEC after exposure to increasing concentrations of cold GABA. Data represent mean±SD of 6 independent experiments. *P<0.05, **P<0.01, and ***P<0.001. GAD indicates glutamic acid decarboxylase.
downstream metabolite) was responsible for the increase in pyruvate oxidation (Figure 5B).

**GABA Promoted Mitochondrial Free Fatty Acid Uptake and Oxidation**

Several reports indicate the important role of fatty acid oxidation toward the maintenance of cellular homeostasis in HUVEC. Therefore, we assessed whether GABA promoted fatty acid oxidation in HUVEC. As fatty acid oxidation is critically regulated at the level of its mitochondrial entry, we investigated whether GABA modulated both uptake and subsequent oxidation of fatty acids in HUVEC. We observed that GABA added to homogenates of HUVEC containing intact mitochondria significantly enhanced the mitochondrial uptake and associated oxidation of [1-14C]palmitate in a concentration-dependent manner (Figure 6A and 6B). [1-14C]palmitate uptake increased by ≈2.5-fold when exposed to a range of 0 to 8 μmol/L GABA. At 10 μmol/L GABA, there was a decrease in the radioactive counts in mitochondrial pellets. [14C]CO2 increased by ≈3.5-fold over a concentration range of 0 to 10 μmol/L GABA. GABA-mediated oxidation of palmitate was observed even in the presence of Gc, confirming that GABA per se (and not a downstream metabolite), was responsible for the increase in fatty acid oxidation (Figure 6C).

**Endogenous GABA Exhibited Anti-Inflammatory Activity in Endothelial Cells**

We next proceeded to explore the modulating role of GABA, if any, on monocyte adhesion either in the basal state or that after endothelial activation by tumor necrosis factor (TNF)-α as occurs in inflammation. We observed that an increase (induced by exposure to Gc) and decrease (induced by exposure to LAG) in the endogenous levels of GABA were associated with a ≈30% decrease and ≈50% increase in monocyte adhesion under basal conditions.

![Figure 5](image1)

**Figure 5.** A, Estimation of [1-14C]pyruvate oxidation by measuring [14C]-labeled acid soluble metabolites (ASM) and 14CO2 produced by the homogenates of human umbilical vein endothelial cells (HUVECs) after exposure to increasing concentrations of gamma aminobutyric acid (GABA). B, Estimation of [1-14C]pyruvate oxidation by measuring [14C]-labeled ASM and 14CO2 produced by the homogenates of HUVEC either at the basal state or in the presence of 4 μmol/L GABA and 45 μmol/L gabaculine (Gc). Data represent mean±SD of 6 independent experiments. *P<0.05, **P<0.01, and ***P<0.001.

![Figure 6](image2)

**Figure 6.** A, Estimation of [1-14C]palmitate uptake in mitochondrial pellets from human umbilical vein endothelial cell (HUVEC) homogenates (containing intact mitochondria) after exposure to increasing concentrations of gamma aminobutyric acid (GABA). B, Estimation of total mitochondrial oxidation of [1-14C]palmitate oxidation by measuring [14C]-labeled acid soluble metabolites (ASM) and 14CO2 produced by the homogenates of HUVEC after exposure to increasing concentrations of GABA. C, Estimation of [1-14C]palmitate oxidation by measuring [14C]-labeled ASM and 14CO2 produced by the homogenates of HUVEC either at the basal state or in the presence of 4 μmol/L GABA and 45 μmol/L gabaculine (Gc). Data represent mean±SD of 6 independent experiments. *P<0.05, **P<0.01, and ***P<0.001.
conditions, respectively. Treatment with TNFα, utilized as a positive control, elicited an increase of ≈220% in monocyte adhesion (Figure 7A). We next tested whether endogenous GABA regulated monocyte adhesion induced by TNFα. We observed that an increase in endogenous GABA (induced by exposure to Gc) elicited a ≈30% decrease in TNFα-induced monocyte adhesion. Conversely a decrease in the levels of endogenous GABA (induced by LAG) was associated with a further increase of ≈25% in TNFα-induced monocyte adhesion (Figure 7B). Silencing of both isoforms of GAD (GADsh) in HUVEC also elicited a similar increase in adhesion both at the basal level and when exposed to TNFα and lipopolysaccharide, compared with Scr. Exogenous GABA reversed the effect of GAD silencing both under basal conditions and when exposed to inflammatory stimulants (Figure 7C). We further investigated whether endogenous GABA regulated the expression of key inflammatory genes associated with the mechanism of monocyte adhesion. Immunoblot analysis revealed that a decrease in endogenous GABA levels (induced by LAG) enhanced the mRNA and protein expression of key adhesion molecules, MCP1 and VCAM1 (Figure 7D). We also compared the mRNA expressions of VCAM1 and MCP1 over time after TNFα treatment between Scr and GADsh. Results indicated that the expression of VCAM1 peaked at 4 hours in both Scr and GADsh although GADsh exhibited significantly higher levels of VCAM1 and MCP1 expressions when compared with

Figure 7. A, Percent change in monocyte adhesion indicated by % change in fluorescence derived from monocytes attached to human umbilical vein endothelial cell (HUVEC) after exposure to gabaculine (Gc), L-allyglycine (LAG), and 10 ng/mL tumor necrosis factor (TNF)-α under flow conditions. B, Percent change in monocyte adhesion indicated by % change in fluorescence derived from monocytes attached to HUVEC pretreated with 10 ng/mL TNFα after exposure to Gc, LAG, and 10 ng/mL TNFα. C, Left, Comparison of monocyte adhesion in scrambled control (Scr), GADsh, and GADsh HUVEC treated with exogenous GABA in basal state and after treatment with TNFα under flow conditions. C, Right, Comparison of monocyte adhesion in Scr, GADsh, and GADsh HUVEC treated with exogenous GABA in basal state and after treatment with lipopolysaccharide under flow conditions. D, Fold change in the mRNA levels of monocyte chemoattractant protein (MCP)-1 and vascular cell adhesion molecule (VCAM) 1 as determined by quantitative polymerase chain reaction in HUVEC with or without treatment with LAG for 24 h. Data represent mean±SD of 3 independent experiments. D, Right, Immunoblot analysis of VCAM1 and MCP1 in lysates of untreated HUVEC (Ctrl) compared with lysates after treatment with LAG. GAPDH is used as the loading control. Data are representative of three experiments. E, Time course of VCAM1 and MCP1 mRNA expressions after TNFα treatment of Scr and GADsh HUVEC. Data represent mean±SD of 3 independent experiments. *P<0.05, **P<0.01, and ***P<0.001. GAD indicates glutamic acid decarboxylase.
Scr. After treatment with TNFα, MCP1 expression in GADsh peaked earlier (at 4 hours) compared with Scr controls (at 8 hours; Figure 7E). This is in accordance with the increase in monocyte adhesion after silencing of GAD under basal condition. Hence, elimination of GABA in endothelial cells led to either an enhanced response to inflammation, as evident from VCAM1 mRNA expression or an earlier onset of inflammatory responses, as evident from MCP1 mRNA expression.

TNFα induces reactive oxygen species, mainly H2O2-mediated inflammation in endothelial cells. Hence, we investigated whether GABA-induced attenuation of TNFα-mediated inflammation was also associated with the down-regulation of H2O2. We observed that in the absence of any external stimuli, an increase (induced by Gc) and a decrease (induced by LAG) in the endogenous levels of GABA by itself resulted in a significant decrease and increase, respectively, in the levels of Amplex red–derived fluorescence (indicating H2O2 levels) in HUVEC (Figure 8A). The assay was validated by the treatment with TNFα (a known enhancer of reactive oxygen species) that increased the levels of Amplex red–derived fluorescence by ≈130% in HUVEC under normal homeostatic conditions (Figure 8A). We further observed that an increase in the endogenous levels of GABA inhibited TNFα-mediated increase in reactive oxygen species by ≈25% and conversely pretreatment with LAG further increased TNFα-mediated enhancement in Amplex red–derived fluorescence by ≈15% (Figure 8B). This clearly indicated that GABA-mediated downregulation of H2O2 was more important under conditions of inflammation, such as exposure to TNFα. Similarly, silencing of both isoforms of GAD (GADsh) in HUVEC elicited a significant increase in H2O2 production (as evident from enhanced Amplex Red fluorescence) both at the basal level and when exposed to TNFα, compared with Scr (Figure 8C, left). Similar to monocyte adhesion, after treatment with TNFα, the percentage change in H2O2 in GADsh cells was significantly higher than that in Scr cells (Figure 8C, right).

**Effect of GABA on Endothelium Intact and Endothelium Denuded Rat Aortic Rings**

GABA had no relaxant or contractile effects on either endothelium intact or endothelium denuded aortic rings (Online Figure VIIIa).

**Effect of GABA on Blood Pressure in Rats**

When GABA at increasing dose were injected intravenously either in the presence or absence of L-NAME, a dose-dependent decrease in BP was recorded (Online Figure VIIIb, left), thereby indicating that the decrease in BP observed after administration of GABA was not indirectly because of release

![Figure 8](http://circres.ahajournals.org/)

**Figure 8.** A, Percent change in Amplex red fluorescence derived from human umbilical vein endothelial cell (HUVEC) after exposure to gabaculine (Gc), L-allylglycine (LAG), tumor necrosis factor (TNF)-α. B, Percent change in Amplex red fluorescence–derived from HUVEC pretreated with 10 ng/mL TNFα after exposure to Gc, LAG. Data represent mean±SD of 3 independent experiments. C, Left, H2O2 production measured by Amplex red fluorescence in basal and TNFα-treated scrambled control (Scr) and GADsh HUVEC. Pegylated catalase (PEG-CAT) used as a negative control. C, Right, Comparison of % change in the increase of H2O2 production after TNFα treatment in Scr and GADsh. *P<0.05, **P<0.01, and ***P<0.001. GAD indicates glutamic acid decarboxylase.
of nitric oxide by GABA. On the contrary, the dose-dependent decrease in BP after intravenous GABA injection was abolished in the presence of the ganglion blocker, Pentolinium (Online Figure VIIIb, right). This indicated that the blood pressure regulatory effect was not because of a direct relaxation of blood vessels by GABA but most likely because of an effect of GABA on the autonomic ganglia similar to that reported by others, and not because of a direct effect of GABA acting through the brain as the concentration of GABA utilized in these studies do not cross the blood–brain barrier.

### Discussion

The primary objective of this study was to assess whether human endothelial cells have the capacity to synthesize and release GABA, which may have important biological actions and may also potentially explain the presence of GABA in the systemic circulation. HAEC and HUVEC were utilized for this purpose. We utilized early passages for our study. We observed that both HAEC and HUVEC had the capacity to synthesize GABA from [1-14C]glutamate. The synthesis of GABA from glutamate by homogenates of both HAEC and HUVEC showed a biphasic response. Initially, there was a concentration-related increase in GABA synthesis by endothelial cell homogenates with increasing concentrations of glutamate (10–50 mmol/L) followed by a decrease at high concentration.

This biphasic nature is not surprising as this is most likely because of product inhibition of the enzyme as described by others. This synthesis of GABA was likely because of the presence of both GAD 65 and 67 in the endothelial cells as demonstrated by immunoblot analysis. We further demonstrated the presence of GABA in endothelial cells utilizing immunofluorescence staining with GABA antibodies. The staining of GABA in the endothelial cells seemed to be both diffuse and in the vesicular form. It has been suggested that the diffuse staining of GABA in neurons is probably because of its synthesis by GAD 67 and that synthesis of GABA stored in vesicles in neurons is synthesized by GAD 65. The presence of GABA in endothelial cells in cross sections of human coronary artery was also observed indicating that the GABA-synthesizing capacity seen in HAEC and HUVEC were a general feature of endothelial cells and not just a feature observed under culture conditions. We utilized both pharmacological inhibition and genetic silencing of both isoforms of GAD in HUVEC to elucidate the effects of GABA. We restrained from using a double knockout mice (mice lacking both GAD 65 and 67) as several lines of evidence indicated that a double knockout mice do not survive >7 days after birth. Utilizing mice with only one of the GAD isoform silenced would have led to complications from the compensatory expression of the other isoform. This was convincingly demonstrated by Asada et al. Soghomonian and Martin, in an in vivo study, where they observed that mice lacking the 65-kDa isoform of GAD (but possessing the intact 67 isoform) maintained normal levels of GABA similar to that seen in the presence of both isoforms. In agreement to this, our data in HUVEC cells demonstrated that individual silencing of either of the GAD isoforms failed to alter the levels of released GABA, whereas simultaneous silencing of both the isoforms significantly lowered the levels of GABA derived from the endothelium.

Moreover, GAD knockout mice would be expected and are in fact compromised in their neuronal activity exhibiting seizures, and in their capacity to produce insulin as it is known that pancreatic β cells produce GABA leading to overall systemic perturbations in neuronal transmissions and metabolism. This would most likely affect endothelial GABA productions, especially when we have observed that GABA produced by endothelial cells regulate metabolism via fatty acid oxidation processes.

We next evaluated whether under steady state conditions, glutamate added to endothelial cells was converted into intracellular GABA. We also wanted to assess whether the GABA synthesized by endothelial cells was only functional intracellularly or whether it was also released outside. The latter may potentially explain another source of GABA detected in the systemic circulation. We observed that [1-14C]glutamate was transported into endothelial cells and converted to [1-14C]GABA and the [1-14C]GABA thus formed was partly retained by the endothelial cells and partly released outside. These results support the concept that human endothelial cells may contribute at least, in part, to the GABA levels reported in the systemic circulation. In the presence of Gc, which inhibits the intracellular inactivation by GABA transaminase, the efflux of GABA from endothelial cells was slightly decreased.

GABA has a stimulatory effect on rat brain mitochondrial ATP synthesis. Therefore, we evaluated whether GABA increased the synthesis of ATP in endothelial cells and potential mechanism(s) involved. We observed that in the presence of LAG, an inhibitor of GABA synthesis, the steady-state basal levels of intracellular ATP decreased, whereas the levels of ATP increased in the presence of Gc. This indicated that GABA synthesized inside the human endothelial cells could increase ATP synthesis. This was further confirmed by the GABA-induced conversion of [8-14C]ADP to [1-14C]ATP. This increase in ATP synthesis in endothelial cells was most likely because of the oxidation of both pyruvate and free fatty acid (FFA). We observed that GABA in a concentration-dependent manner increased [14C]CO2 production from [1-14C]pyruvate in HUVEC homogenates with intact mitochondria. This was because of GABA and not a downstream metabolite of GABA as Gc did not modify the effect. GABA did not induce any increase in the uptake of [1-14C]palmitate by endothelial cells (data not shown) indicating that GABA had no effect on the transport of FFA from the extracellular milieu into the endothelial cells. However, similar to that with pyruvate, we observed that GABA in a concentration-dependent manner significantly increased the uptake and subsequent oxidation of [1-14C]palmitate by mitochondria also leading to an increase in ATP synthesis. Radioactive counts of [1-14C]palmitate in the mitochondrial pellet exhibited progressive increase with increased concentrations of added GABA, except a decrease at the high 10 μmol/L concentration. This can be attributed to the fact that at 10 μmol/L GABA, the half-life of uptake [1-14C]palmitate was significantly less because of its rapid oxidation. The GABA-mediated oxidation of palmitate was also observed in the presence of Gc confirming that GABA and not a downstream metabolite was responsible for the increase in
fatty acid oxidation. It, therefore, appears that an increase in ATP synthesis by mitochondria of HUVEC is because of an increase in oxidation of both pyruvate and FFA.

The stimulatory effect of GABA on FFA oxidation (FFAO) in HUVEC could be of immense physiological significance, especially under conditions of starvation. It has been reported that AMP kinase (stress signal)–activated FFAO accounts for more than ~40% of cellular ATP produced in HUVEC.31 GABA-induced FFAO could also be critical in endothelial cells in the tumor microenvironment, where it has been shown that FFAO is the major source for nucleotide synthesis.32 Furthermore, GABA could be important in regulating endothelial cell permeability, as it has recently been reported that FFAO has emerged as a central regulator of endothelial cell permeability and blood vessel stability.33

Administration of GABA markedly attenuated insulin and systemic inflammation in mice.50 Therefore, we evaluated whether GABA had any anti-inflammatory effects on endothelial cells. We assessed the effects of GABA on both the basal and the TNFα-induced increase in adhesion of monocytes to endothelial cells and the modulating role of GABA, if any, on both VCAM1 and MCP1 expression in this process. We focused on both VCAM1 and MCP1 as both of these are involved in adhesion of monocytes to endothelial cells.50,51 Our results indicated that basal levels of GABA synthesized by endothelial cells had anti-inflammatory activity. This is because LAAG, an inhibitor of GABA synthesis or silencing of both GAD 65 and 67, increased monocyte adhesion and H2O2 production and Gc on its own decreased monocyte adhesion and H2O2 production. Furthermore, when endothelial activation was induced either by TNFα in cells pretreated with LAG or GAD 65/67 silenced HUVEC, there was a significant increase in monocyte adhesion and H2O2 production when compared with the cells treated with TNFα alone. By contrast when TNFα was added to Gc-pretreated endothelial cells, the monocyte adhesion and H2O2 production were significantly decreased when compared with TNFα alone. This decrease in monocyte adhesion to endothelial cells is most likely because of decreased expression of both VCAM1 and MCP1 as observed by us. Expectedly, increased nuclear localization of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) was also observed in GADsh HUVEC after TNFα treatment in comparison with Scr HUVEC. Initial screening of MAP kinase pathways did not elicit any changes on silencing of GAD (Online Figure VI).

Our work may have potential physiological implications. Circulating levels of GABA have been reported in the venous blood of humans,13 and the levels seem to be decreased in preeclampsia.52,53 However, to date, the source of GABA in the peripheral circulation has not yet been fully ascertained. The results from our work indicated that the endothelial cells may be an important source of GABA in the circulation along with that produced by the pancreatic β cells, adrenal gland, and certain immune cells among others. Furthermore, preeclampsia is associated with endothelial dysfunction, hypertension, and an increase in platelet aggregation.54 GABA inhibits aggregation of human platelets,55 and administration of GABA to both humans and laboratory animals at doses that do not cross the blood–brain barrier reduces BP.21,35,36 Our study and those of others indicated that this action of GABA is most likely through its action on the autonomic ganglia. Therefore, the decrease in circulating GABA in preeclampsia may indicate that GABA synthesis in endothelial cells is decreased and may, therefore, be partly responsible for some of the symptoms seen in this condition.50 The fact that the BP-lowering effect of intravenous GABA was abrogated on administration of pentolinium (a ganglionic blocker and an acetylcholine nicotinic receptor antagonist) indicated that GABA could act on acetylcholine nicotinic receptors in the ganglion similar to that observed by others.21,35,36 In this study, we did not elucidate the action of released GABA on endothelial cells themselves. However, we have preliminary results that strongly suggested that exogenous GABA, induced the expression of GABA(A) receptors on HUVEC (Online Figure IV). Studies are underway to further elucidate the role of GABA receptors in the endothelium.

The capillary distributions in the myocardium are dense and are in close proximity to the cardiomyocytes to allow for supply of adequate nutrients.57 A major source of energy for cardiomyocytes is FFA oxidation.56 It is, therefore, possible that GABA released by the endothelium increases FFA oxidation by the cardiomyocytes providing an important source of energy.58 Preliminary studies have actually indicated the presence of GABA in cardiomyocytes in the vicinity of coronary vessels of the heart (Online Figure V). Therefore, the endothelium-derived GABA may have a significant impact in humans like other endothelial vasodilators previously described like prostacyclin and nitric oxide.10–12

Even though our study has identified human endothelial cells as a novel source of GABA, in vivo models need to be explored to further its physiological implications. This limitation will be addressed by future studies utilizing endothelial-specific GAD knockout animal models.

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Disclosures

None.

References


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**Novelty and Significance**

**What Is Known?**

- GABA, an inhibitory neurotransmitter, is found in the systemic circulation at a concentration between 0.5 and 3 μmol/L.
- Certain non-neuronal cells, including the β cells of the pancreas, have been shown to produce GABA. However, the amount released from these cells does not account for the overall circulatory concentrations.

**What New Information Does This Article Contribute?**

- Human endothelial cells can synthesize and release GABA.
- GABA can stimulate fatty acid oxidation and overall endogenous ATP production.
- The endothelium-derived GABA may have an anti-inflammatory role.
\textit{\textbf{\textgamma-\textbf{Aminobutyric Acid Is Synthesized and Released by the Endothelium: Potential Implications}}}

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Supplemental Material

Results:
The optimum concentration of L-Allyl Glycine (LAG) was determined from a concentration response study (Online Fig I). 300μM LAG elicited the maximum inhibition as determined by GAD assay utilizing HUVEC homogenate as mentioned in the main text. There was a biphasic response in GAD activity with concentrations above 300μM LAG eliciting a slight increase. This could be due to non-specific action of LAG at higher concentrations. However other workers as already mentioned in the main text, have used concentrations of 100-300μM similar to ours.
The optimum concentration of gabaculine (GC) was determined utilizing a concentration response study comparing GAD activity in HUVEC homogenate against varying concentrations of GC. The concentration of GC eliciting maximum GABA production was 45 μM (Online Fig II). This was in agreement with earlier reported studies as already mentioned in the main text. Sections of normal adult human heart tissue were obtained from Translational Pathology Core Laboratory, UCLA. GAD65/67 was detected in the tissue sections, utilizing rabbit polyclonal anti-GAD67/67 antibody in 1:500 dilutions, as per manufacturer’s instruction (Online Fig III).
We also observed the induction of the mRNA expressions of GABA_Aρ1, GABA_Aρ2 and GABA_Aρ3 receptors upon exposure to 4μM GABA for 2hrs. This may indicate that exogenous GABA may signal through chloride dependent ion channels in the endothelium (Online Fig IV). Interestingly, a strong signal for GABA was observed in the cardiomyocytes surrounding coronary vessels. It may indicate the GABA is released from coronary vessels to the surrounding cardiomyocytes (Online Fig V).
ELISA based screening for activation of MAP kinases did not elicit changes upon GAD silencing (Online Fig VI a). Increased nuclear localization of NFkB was observed in GADsh HUVEC following TNFα treatment in comparison to Scr HUVEC (Online Fig VI b). Individual silencing of either GAD 65 or GAD 67 did not alter the levels of released endothelial derived GABA. However silencing of both GAD 67 and GAD 67 together significantly lowered levels of released GABA (Online Fig VII).
GABA had no relaxant or contractile effects on either endothelium intact or endothelium denuded aortic rings (Online Fig VIII a).
When GABA at increasing dose were injected intravenously either in the presence or absence of L-NAME, a dose dependent decrease in BP was recorded (Online Fig VIII b, left panel) thereby indicating that the decrease in BP observed following administration of GABA was not indirectly due a release of NO by GABA. On the other hand, the dose-dependent decrease in BP following intravenous GABA injection was abolished in presence of the ganglion blocker, Pentolinium (Online Fig VIII b, right panel). This indicated that the blood pressure regulatory effect was not due to a direct relaxation of blood vessels by GABA but most likely due to an effect of GABA on the autonomic ganglion similar to that reported by others (1-3) and not due to a direct effect of GABA acting through the brain as the concentration of GABA utilized in these studies do not cross the blood brain barrier (4,5).
Online Figure I: Concentration dependent inhibition of GABA production by HUVEC homogenates (150μg) by various concentrations of LAG (L-Allyl Glycine) in the presence of excess glutamate. GABA production was determined by the ELISA based immune assay as described in the main text. *p<0.05, **p<0.01, ***p< 0.001
Online Figure II: Concentration dependent increase in GABA production by HUVEC homogenates (150μg) by various concentrations of Gabaculine (GC) in the presence of excess glutamate. GABA production was determined by the ELISA based immune assay as described in the main text. *p<0.05, **p<0.01, ***p< 0.001
Online Figure III: Detection of GAD65/67 in the endothelial lining of vessels from human heart tissue section (indicated by brown staining).
Online Figure IV: Induction of the mRNA expressions of GABA_{A\rho1}, GABA_{A\rho2} and GABA_{A\rho3} receptors upon exposure to GABA.
Online Figure V: Detection of GABA (indicated by brown staining) in the cardiomyocytes adjacent to the coronary vessels
**Online Figure VI:** (a) Screening of MAP kinase activation in Scr and GADsh utilizing ELISA based methods (b) Nuclear localization of NFκB in GADsh and Scr HUVEC following TNFα treatment. Blue indicates nuclear staining and Green indicates the p65 subunit of NFκB.
Online Figure VII: (a) mRNA expression of GAD 1 (67) and GAD 2 (65) following individual or dual silencing (b) GABA released from either single GAD isoform silencing or from silencing of both the isoforms of GAD, in HUVEC.
Online Figure VIII: (a) Percentage relaxation of isolated aortic rings from rat with or without endothelial cells (EC) with increasing concentrations of acetylcholine (Ach) (left panel) and GABA (right panel). (b) Blood pressure lowering effect of GABA in anesthetized rats (N=8) in presence or absence of L-NAME (left panel) and a ganglion blocker Pentolinium (right panel). Results depicted as mean±SD.
References:


