Thyrotropin-releasing hormone overexpression induces structural changes of the left ventricle in the normal rat heart

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Thyrotropin-releasing hormone (TRH) overexpression induces structural changes of the left ventricle in the normal rat heart. Am J Physiol Heart Circ Physiol 307: H1667–H1674, 2014. First published October 3, 2014; doi:10.1152/ajpheart.00494.2014.—Thyrotropin-releasing hormone (TRH) hyperactivity has been observed in the left ventricle of spontaneously hypertensive rats. Its long-term inhibition suppresses the development of hypertrophy, specifically preventing fibrosis. The presence of diverse systemic abnormalities in spontaneously hypertensive rat hearts has raised the question of whether specific TRH overexpression might be capable of inducing structural changes in favor of the hypertrophic phenotype in normal rat hearts. We produced TRH overexpression in normal rats by injecting into their left ventricular wall a plasmid driving expression of the preproTRH gene (PCMV-TRH). TRH content and expression of preproTRH, collagen type III, brain natriuretic peptide, β-myosin heavy chain, Bax-to-Bcl-2 ratio, and caspase-3 were measured. The overexpression maneuver was a success, as we found a significant increase in both tripeptide and preproTRH mRNA levels in the PCMV-TRH group compared with the control group. Immunohistochemical staining against TRH showed markedly positive brown signals only in the PCMV-TRH group. TRH overexpression induced a significant increase in fibrosis, evident in the increase of collagen type III expression accompanied by a significant increase in extracellular matrix expansion. We found a significant increase in brain natriuretic peptide and β-myosin heavy chain expression (recognized markers of hypertrophy). Moreover, TRH overexpression induced a slight but significant increase in myocyte diameter, indicating the onset of cell hypertrophy. We confirmed the data “in vitro” using primary cardiac cell cultures (fibroblasts and myocytes). In conclusion, these results show that a specific TRH increase in the left ventricle induced structural changes in the normal heart, thus making the cardiac TRH system a promising therapeutic target.

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heart tissue, these results raise a number of questions, including a very important one: is the single upregulation of TRH expression and activity a sufficient step predisposing LV fibrosis as well as other structural changes in the normal heart?

Thus, we speculated that specific LV TRH overexpression could be able to induce fibrosis and maybe hypertrophic features in normal hearts.

**MATERIALS AND METHODS**

**Ethics statement.** The Institutional Animal Care and Use Committee approved the animal experimentation protocols following their ethical guidelines. The protocol was approved by CICUAL UE-IDIM (IDIM-CONICET, Buenos Aires, Argentina). All protocols were performed under anesthesia, and all efforts were made to minimize suffering.

All reagents were from Sigma (St. Louis, MO) unless otherwise indicated.

**Animals.** Eight-week-old male Wistar rats (10 rats/group, Charles River Laboratories) were used. Animals were housed in a room with controlled temperature (23 ± 1°C) under a 12:12-h light-dark schedule.

*In vivo* TRH overexpression. The pcDNA3 plasmid (5.4 kb) was used (Invitrogen). Rat TRH cDNA was cloned downstream of the strong cytomegalovirus promoter (PCMV-TRH). The control group received empty pcDNA3 (PCMV-Con). Rats were assigned in a random blind fashion to one of two groups, PCMV-TRH or PCMV-Con (n = 10 rats/group), and anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg). Previously, we performed a dose curve (25, 50, and 100 μg plasmid/injection), and we used 100 μg, which showed the maximal induction. In our hands, PCMV transfection induced TRH expression during >7 days; indeed, injections were conducted weekly during the experiment (4 wk) to maintain the induction. All injections were performed under echography. The chests of the rats were shaved and cleaned. Gel was liberally applied to the exterior body surface before placement of the probe. The probe was aligned with the needle, and special care was taken to visualize the region of the myocardial wall. Injection was performed in three different sites (from the base to apex) to efficiently surround the entire LV as previously described (23, 16).

All experiments were also performed blindly with respect to the treatment. At the end of the protocol, rats were decapitated, and hearts were removed.

**Isolation and culture of neonate rat ventricular myocytes and fibroblasts.** We used neonatal cell culture to confirm “in vitro” TRH overexpression results, as this has been vastly reported has several advantages for studies examining cellular response, among them, cultured neonatal cells can be more easily transfected than adult cells. Wistar neonatal rat ventricular myocytes and fibroblasts were isolated and prepared as previously described (22). Briefly, myocytes were dispersed from ventricles of 1- to 3-day-old Wistar rats by digestion with collagenase type II and trypsin at 37°C (GIBCO). The cell suspension was separated on a discontinuous Percoll gradient to obtain myocardial cell cultures with >95% myocytes. Myocytes were preplated in 10-cm culture dishes in DMEM containing 100 μg/ml streptomycin and 100 U/ml penicillin supplemented with 10% FBS for 1–2 h to remove nonmyocyte cells. Unattached myocytes were removed, and equal numbers of cells were plated on 35-mm collagen type I-coated plates. To inhibit noncardiac myocyte growth, 100 μmol/l bromodeoxyuridine was also added.

After the Percoll gradient, the layer of fibroblasts was plated in 10-cm culture dishes in DMEM containing 100 μg/ml streptomycin and 100 U/ml penicillin supplemented with 10% FBS.

In all experiments, myocytes or fibroblasts were deprived of serum and incubated for another 24 h before treatment.

**Cell transfection.** Transfection of cardiac cells was performed using Lipofectamine 2000 (Invitrogen) based on the manufacturer’s recommendations. For optimization of the procedure, the ratios of Lipofectamine to PCMV plasmid DNA concentration (in μl/μg) were selected to get the best result (5/1). Expression of TRH was evaluated using quantitative real-time RT-PCR.

**TRH content.** For peptide extraction, cardiac tissue samples were collected in acetic acid (2 mol/l) and HCl (0.1 mol/l). All samples were boiled for 20 min and centrifuged at 5,000 g, and the supernatant was lyophilized. The residue was dissolved in the appropriate buffer for the measurement of TRH-like immunoactivity by RIA or the mobile phase for the identification of authentic TRH by HPLC. This procedure and the RIA for TRH have been previously reported in detail (6).

**Quantitative real-time RT-PCR.** Quantification of preproTRH, TRH receptor 1, prohormone convertase 1 (PC1), collagen type III, BNP, atrial natriuretic peptide (ANP), and caspase-3 mRNA expression was performed using a real-time RT-PCR technique normalized by β-actin housekeeping gene expression. Briefly, for cDNA synthesis, 2 μg of total RNA (from tissue or cells) and 1 μg of random primer (Lipidofectamine) were used (Invitrogen) based on the manufacturer’s recommendations. For optimization of the procedure, the ratios of Lipofectamine to PCMV plasmid DNA concentration (in μl/μg) were selected to get the best result (5/1). Expression of TRH was evaluated using quantitative real-time RT-PCR.

Fig. 1. Left ventricular (LV) thyrotropin-releasing hormone (TRH) overexpression in Wistar rats. A–C: LV TRH content (in pg/mg protein) measured by RIA (A) and TRH precursor (preproTRH; B) and prohormone convertase 1 (PC1; C) gene expression determined by real-time PCR normalized by β-actin expression in Wistar rats injected with the PCDNA3 plasmid encoding the preproTRH precursor gene (PCMV-TRH) or the empty PCDNA3 plasmid used as a control (PCMV-Con). n = 6 rats/group. *P < 0.04 between groups.
Fig. 2. Effect of LV TRH overexpression on fibrotic and hypertrophic markers. A and B: collagen type III (A), brain natriuretic peptide (BNP; B) and β-myosin heavy chain (β-MHC; B) mRNA expression were determined by real-time PCR and normalized by β-actin expression. Results are expressed as percentages of the control group; n = 6 rats/group. *P < 0.03 compared with the PCMV-Con group.

Table 1. Effect of LV TRH overexpression on morphometric variables

<table>
<thead>
<tr>
<th>Morphometric Variables</th>
<th>PCMV-TRH Group</th>
<th>PCMV-Con Group</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV wall</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiomyocyte diameter, μm</td>
<td>26.3 ± 0.7</td>
<td>23.8 ± 0.6</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>Cardiomyocyte density, number of cardiomyocytes/area</td>
<td>11.5 ± 0.3</td>
<td>12.8 ± 0.4</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Number of capillaries/area</td>
<td>25.6 ± 0.5</td>
<td>28.1 ± 0.6</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Myocyte-to-capillary ratio</td>
<td>0.44 ± 0.03</td>
<td>0.45 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>IVS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiomyocyte diameter, μm</td>
<td>26.4 ± 0.7</td>
<td>23.6 ± 0.6</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Cardiomyocyte density, number of cardiomyocytes/area</td>
<td>11.4 ± 0.3</td>
<td>12.8 ± 0.3</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>Number of capillaries/area</td>
<td>25.4 ± 0.6</td>
<td>27.9 ± 0.5</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Myocyte-to-capillary ratio</td>
<td>0.44 ± 0.06</td>
<td>0.45 ± 0.06</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SE. LV, left ventricular; TRH, thyrotropin-releasing hormone; PCMV-TRH group, group injected with the PCDNA3 plasmid encoding the preproTRH precursor gene; PCMV-Con group, group injected with the empty PCDNA3 plasmid (control group); IVS = interventricular septum; NS, not significant.
(vessels < 8 µm) within the confines of each of the 20 random views at ×400 magnification.

Biochemical and pathology experiments. At the end of the treatment, rats were weighed, and the snout-tail long index was measured before rats were killed by decapitation. Blood samples were collected with sodium EDTA, and thyroid hormone levels were measured using an enzyme immunometric assay (Assay Designs). Hearts were rapidly removed and weighed. In some animals, cardiac tissues were separated (atria, LV, right ventricle, and septum) for TRH determination by RIA and for the quantification of mRNA expression; others were used for pathology experiments.

Systolic arterial blood pressure measurement. Animals were acclimated in a quiet room for 30 min before the measurement of systolic arterial blood pressure (SABP) by a tail-cuff method weekly during the experiment.

Statistics. Values are expressed as means ± SE. All statistical analyses were performed using absolute values and processed using Statistics (version 6.0, Software). The assumption test to determine Gaussian distribution was performed by the Kolmogorov-Smirnov method. For parameters with Gaussian distribution, comparisons between groups were carried out using one-way ANOVA followed by Bonferroni’s test, Kruskal-Wallis test (nonparametric ANOVA), and Dunn’s multiple comparison test when appropriate. In the case of two groups, comparison was performed using a t-test or Mann-Whitney test when appropriate. P values of <0.05 were considered significant.

RESULTS

Following the described protocol, we found significant increases in both TRH content (nearly 2-fold) and preproTRH mRNA expression (>3-fold) in the LV of animals injected with PCMV-TRH with respect to control rats injected with the empty plasmid (PCMV-Con, P < 0.04), indicating the effectiveness of the LV TRH overexpression design (Fig. 1, A and B). We also tested the rest of the heart chambers and found no significant changes in the right ventricle and atria, although a small increase was also seen in the septum compared with the PCMV-Con group, which did not reach statistical significance. In contrast, although there was a tendency, no significant differences were observed in PC1 gene expression between the groups (Fig. 1C), even though Perello et al. (20) have reported that PC1 is a specific convertase involved in proTRH processing as a regulator step in TRH activity in many tissues.

To confirm the specificity of TRH overexpression, preproTRH gene expression was evaluated in other heart tissues of...
the same animals such as the diencephalon, because it is known that the central TRH system participates not only in the endocrine system but also in cardiovascular regulation (6), as well as in the pancreas, where the tripeptide has also been found (19). No differences were observed between the two groups, indicating the specificity of the treatment in inducing LV TRH overexpression [diencephalic preproTRH: 100 ± 17 arbitrary units in the PCMV-Con group vs. 142 ± 22 arbitrary units in the PCMV-TRH group and pancreatic preproTRH: 100 ± 11 arbitrary units in the PCMV-Con group vs. 90 ± 25 arbitrary units in the PCMV-TRH group, not significant (NS)]. Accordingly, at the end of the experiment, thyroid hormones were also evaluated in both groups of animals and no differences were found. These normal ratios found in animals injected with PCMV-TRH were probably due to different magnitudes of the TRH effects on the fibrotic (strong) and hypertrophic (slight) processes. In addition, we cannot discard that the short period of the treatment (4 wk) was not sufficient to reveal ongoing macroscopic changes and that only microscopic changes were evident.

To further confirm the structural changes induced by LV TRH overexpression, histological experiments of the LV in

![Image](http://ajpheart.physiology.org/)

**Fig. 2.** A: graph showing the expression of Bax/Bcl2 in LV of PCMV-Con and PCMV-TRH groups. B: representative images of caspase-3 expression in LV sections of PCMV-Con and PCMV-TRH groups.

**Table 1.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PCMV-Con</th>
<th>PCMV-TRH</th>
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<tbody>
<tr>
<td>SABP (mmHg)</td>
<td>126 ± 11</td>
<td>130 ± 15</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>380 ± 22</td>
<td>355 ± 42</td>
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</tbody>
</table>

During all experiments, SABP and body weight were measured weekly, and we did not find differences between the groups (SABP: 126 ± 11 mmHg in the PCMV-Con group vs. 130 ± 15 mmHg in the PCMV-TRH group and heart rate: 380 ± 22 beats/min in the PCMV-Con group vs. 355 ± 42 beats/min in the PCMV-TRH group, NS). Thus, it is unlikely that the structural changes observed could be consequence of an increase in arterial blood pressure.

At the end of the experiment, two additional hypertrophy indexes were measured (heart weight/body weight and heart weight/snout-tail long indexes), for which no significant differences were found. These normal ratios found in animals injected with PCMV-TRH were probably due to different magnitudes of the TRH effects on the fibrotic (strong) and hypertrophic (slight) processes. In addition, we cannot discard that the short period of the treatment (4 wk) was not sufficient to reveal ongoing macroscopic changes and that only microscopic changes were evident.

To further confirm the structural changes induced by LV TRH overexpression, histological experiments of the LV in
both groups were performed. As shown in Fig. 3, evaluation of ECM expansion was performed using Masson’s trichrome (A and B) and Azan-Mallory staining (E and F). The results showed that LV TRH overexpression induced a significant and important \( P < 0.01 \) increase in ECM expansion in normal Wistar rats. Furthermore, in agreement with the observation of elevated collagen type III mRNA expression in the LV in the TRH-overexpressing group, a substantial increase \( P < 0.01 \) in collagen, as evaluated by Sirius red staining, was observed (Fig. 3, C and D).

Finally, in accordance with TRH mRNA expression and tripeptide content data, immunohistochemical analysis of the LV using a specific antibody against TRH (7) showed a positive brown signal in the PCMV-TRH group with respect to the PCMV-Con group (Fig. 3, G and H), confirming that in vivo overexpression was mainly induced in cardiomyocytes.

Since it is known that ECM expansion occurs after myocyte cell death, we hypothesized that PCMV-TRH group would present higher apoptotic indexes. In that sense, we evaluated apoptosis markers and measured the proapoptotic-to-antiapoptotic (Bax-to-Bcl-2) ratio (Fig. 4A), caspase-3 expression, and presence of caspase-3 by immunostaining (Fig. 4B). Although the Bax-to-Bcl-2 ratio did not show any differences between the two groups, we observed a significant \( P < 0.03 \) increase in caspase-3 expression in the PCMV-TRH group. Indeed, we found an increase in the number of cells with positive staining for caspase-3 (by the immunoperoxidase technique) in the PCMV-TRH group (6.2 + 1.1 nuclei/area) with respect to the PCMV-Con group (0.6 + 0.7 nuclei/area, \( P < 0.01 \)), indicating an apoptotic induction in these animals.

Finally, trying to examine which cell type was involved in TRH-induced effects, we tested TRH overexpression in primary Wistar rat cell cultures (myocyte and fibroblasts). We transitory transfected cardiac cells and measured TRH overexpression effects on gene expressions 24 and 96 h posttreatment.

As shown in Fig. 5A, TRH overexpression in myocytes was achieved 24 h after transfection, given that augmented TRH precursor expression was evident in the PCMV-TRH group. As expected, this overexpression was transitory, because 96 h after treatment, TRH precursor expression was similar to the control group. In agreement with the in vivo experiments, the TRH increase significantly induced the expression of BNP and \( \beta \)-MHC at 24 h (Fig. 5, B and C). Moreover, an induction of the apoptotic index (Bax/Bcl-2) was manifest at 24 h, when TRH overexpression was maximal.

Furthermore, as shown in myocytes, cardiac fibroblasts showed efficient TRH overexpression at 24 h posttransfection that provoked a significant increase in collagen type III expression 48 h after treatment, which was maximal at 96 h, the last time point studied (Fig. 6). These observations are in accordance with our knowledge of cardiac physiology, as myocyte death occurs first, followed by the ECM activation triggered by the collagen increase being fibrosis, the final stage of this process.

**DISCUSSION**

In 1992, a cardiac TRH system was reported, and, since then, many authors have studied the role of local TRH in cardiac physiology (1, 9, 26). An increase in TRH expression in the ventricle of infarcted rats after an 8-wk injury has been reported, suggesting the participation of the tripeptide in the cardiac damage (11). Afterward, our group showed that adult SHRs presented LV TRH system hyperactivity. We then dem-

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**Fig. 5.** Effect of TRH transfection on hypertrophic and apoptotic marker expression in myocyte primary cell cultures from Wistar neonates. mRNA expression was determined by real-time PCR and normalized by \( \beta \)-actin expression. A–D: TRH precursor expression (A), BNP expression (B), \( \beta \)-MHC expression (C), and apoptotic ratio (Bax/Bcl-2; D). Results are expressed as percentages of the control group. \( n = 5 \) rats/group. *\( P < 0.05 \), cells transfected with PCMV-TRH compared with cells transfected with PCMV-Con.
onstrated that long-term inhibition of the TRH system in young SHRs prevented cardiomyocyte enlargement, ECM expansion, and fibrosis development. Moreover, no differences were seen at the adult stage between Wistar-Kyoto rats and SHRs treated with specific TRH siRNA, indicating that the treatment was efficient and avoided detrimental TRH actions on cardiac tissue (23).

To our knowledge, all evidence on the role of cardiac TRH in heart damage has been obtained in systems where other abnormalities were concurrent (infarcted rats, SHRs, etc.) (11, 12). In this regard, the SHR model has been extensively studied, and the participation of many systems in LV hypertrophy development has been described (the renin-angiotensin system, endothelin system, adrenergic system, etc.) (2, 23, 18).

On this basis, we speculated whether single ventricle TRH overexpression would be enough to induce cardiac structural changes in a normal rat independent of any other alteration. Results are expressed as percentages of the control.

LV TRH overexpression was efficiently induced in normal hearts of Wistar rats injected with PCMV-TRH compared with control rats injected with PCMV-Con. PCMV-TRH has been previously used to induce central TRH overexpression (8) and other proteins in cardiac tissue using the same technique. LV TRH overexpression was specific, as we did not find TRH expression changes in other tissues (brain and pancreas). Indeed, both groups showed similar plasma thyroid hormone levels, and the in vivo intracardiac treatment, which involved repeated injections of the plasmid, did not produce body weight or SABP changes throughout the experiment.

As expected, TRH overexpression for 4 wk was not enough to induce a 10-fold TRH increase, as has been observed in adults SHR (23); however, a twofold TRH increase was able to induce expression of fetal genes and fibrosis, suggesting that chronic LV TRH could lead to the development of fibrosis and hypertrophy in a normal rat.

In that sense, this long-term LV TRH overexpression induced an increase in the hypertrophy marker BNP that was accompanied by a smaller increase in ANP expression, indicating structural changes focused to a hypertrophic phenotype shift of the heart tissue. Accordingly, we found a significant increase in β-MHC and collagen type III expression that was confirmed by histological experiments in which an increase in myocyte diameter and ECM protein expression were observed only in the LV in the TRH-overexpressing group.

On the other hand, many authors have described that the first structural changes, which include a reduction in myocyte mass and associated functional loss, are due to increased cell death by apoptosis (14, 21). Therefore, increases in proapoptotic Bax and caspase-3 proteins have been described in hypertrophied LVs of young and adult SHRs, and, as we have shown in previous work, long-term inhibition of LV TRH ameliorates not only heart hypertrophy but also reduces the exaggerated apoptosis that accompanies heart failure in this model (23). On this basis, as a first step, we evaluated the apoptosis ratio (Bax/Bcl-2) as an index of cells’ susceptibility to apoptosis and measured caspase-3 RNA and protein levels. We found a significant increase (P < 0.03) in caspase-3 expression only in the PCMV-TRH-injected group, although Bax/Bcl-2 did not show any difference at the end of the experiment. Moreover, we evaluated the caspase-3 signal by an immunoperoxidase technique and found a significant increase in caspase-3 in the TRH-overexpressing group, confirming the increased activity of a caspase-3-mediated apoptotic pathway in these hearts.

Our results are the first demonstration that, in a normal heart, the increase in LV TRH expression alone could induce structural changes that are shared with features of hypertrophy. These observations confirm that cardiac TRH induces a heart shift, in which structural alterations such as fibrosis, hypertrophy, and apoptosis could be involved, independently of any other system alterations.

Finally, we developed experiments using primary cardiac cell cultures to try to confirm TRH-induced effects in vitro. As expected, TRH overexpression was successfully induced in both cell types 24 h after plasmid transfection, confirming the presence of TRH biosynthesis machinery in either myocytes or fibroblasts. We extended the experiment up to 96 h, when the acute TRH overexpression effect was negligible. The difference in the time course with the in vivo experiment is probably due to the experimental procedure (transfection cell efficiency, cell division, etc.), as discussed below.
However, as hypothesized, TRH overexpression was capable to induce expression of hypertrophic markers BNP and β-MHC, effects that reach a peak at 24 h.

In agreement with the notion that hypertrophy is accompanied by myocyte cell death, we found increased apoptotic index expression at 24 h, when TRH overexpression reached its maximum, indicating that TRH overexpression in myocyte cells is involved in hypertrophic and apoptotic processes.

In parallel, we studied the effects of TRH overexpression in cardiac fibroblasts, and, unlike what we observed in myocytes, TRH induction triggered a significant increased in collagen type III expression that was maximal (5-fold) at 96 h, indicating that TRH participates in ECM expansion the latest stage.

It is well known that primary cell culture transfection did not respond as in vivo transfection; indeed, we found discrepancies between TRH overexpression, i.e., induced effect time course, limiting the comparison between in vitro and in vivo results. Nevertheless, as a whole, the cell culture results confirmed a TRH direct action on cardiac cells.

Finally, both in vitro and in vivo results were in agreement with our previous publication (23), in which disruption of the cardiac TRH system impeded fibrosis and hypertrophy in SHRs, and outstand the contribution of the TRH system in the cardiac TRH system impeded fibrosis and hypertrophy in spontaneously hypertensive rats.

Interestingly, several cardiac pathologies have shown increased cardiac fibrosis, with ECM expansion, myocyte hypertrophy, and apoptosis; examples include infarct and obesity with hyperleptinemia, where TRH hyperactivity has been observed (13, 5). In these situations where the cardiac TRH system is upregulated, it appears that its inhibition may be protective for the heart.

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D I S C L O S U R E S
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A U T H O R  C O N T R I B U T I O N S

R E F E R E N C E S