Thiorphan-Induced Survival and Proliferation of Rat Thymocytes by Activation of Akt/Survivin Pathway and Inhibition of Caspase-3 Activity

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ABSTRACT
The activity of substance P (SP) in the rat thymus seems to be tightly controlled by its bioavailability. In this study, we provide evidence for the expression of the SP-degrading enzyme, neutral endopeptidase (NEP)/CD10, by rat thymocyte subsets, and we illustrate its involvement in the in vivo SP/neurokinin-1 receptor (NK1R)-mediated regulation of thymocyte survival and proliferation. NEP/CD10 was expressed at both mRNA and protein levels on a substantial portion (45.5%) of CD5- thymocytes, namely on the CD4+CD8− (double positive; DP) and CD4− subsets. Continuous administration of thiorphan, a specific NEP/CD10 inhibitor, by means of miniosmotic pumps, enhanced thymocyte preprotachykinin-A (PPT-A) and NK1R mRNA expression as well as SP and NK1R protein levels in an NK,R-dependent manner. Thiorphan increased CD10+CD4− and CD10+DP thymocyte numbers, and an NK,R antagonist, (S)1-{2-[3(3-4-dichlorophenyl)-1-(3-iso-propoxyphenylacetyl)piperidine-3-yl]ethyl}-4-pheny-1-azoniabicyclo[2.2.2]octane, chloride (SR140333), abrogated these stimulatory effects. In addition, the NEP/CD10 inhibitor stimulated interleukin (IL)-2 production, IL-2 receptor α chain expression, and concanavalin A-induced proliferation of CD5+ thymocytes, and it inhibited spontaneous and NK,R-dependent thymocyte apoptosis. The thiorphan-protective antiapoptotic and proliferative effects involved the activation of Akt serine-threonine kinase, subsequent up-regulation of survivin mRNA, down-regulation of procaspase-3 mRNA levels, and suppression of caspase-3 activity, which were inhibited by SR140333 and mimicked by exogenous SP administration. Overall, our findings suggest that by controlling SP availability, NEP/CD10 negatively regulates thymocyte homeostasis and development.

Development of T lymphocytes is a complex process that depends on both stromal cell interactions and the production of soluble factors such as cytokines, peptide hormones, and neuropeptides locally synthesized or released in the thymic microenvironment (Blalock and Smith, 1985). In the thymus, the concentration of biologically active peptides is mainly controlled by its bioavailability. In this study, we provide evidence for the expression of the SP-degrading enzyme, neutral endopeptidase (NEP)/CD10, by rat thymocyte subsets, and we illustrate its involvement in the in vivo SP/neurokinin-1 receptor (NK1R)-mediated regulation of thymocyte survival and proliferation. NEP/CD10 was expressed at both mRNA and protein levels on a substantial portion (45.5%) of CD5- thymocytes, namely on the CD4+CD8− (double positive; DP) and CD4− subsets. Continuous administration of thiorphan, a specific NEP/CD10 inhibitor, by means of miniosmotic pumps, enhanced thymocyte preprotachykinin-A (PPT-A) and NK1R mRNA expression as well as SP and NK1R protein levels in an NK,R-dependent manner. Thiorphan increased CD10+CD4− and CD10+DP thymocyte numbers, and an NK,R antagonist, (S)1-{2-[3(3-4-dichlorophenyl)-1-(3-iso-propoxyphenylacetyl)piperidine-3-yl]ethyl}-4-pheny-1-azoniabicyclo[2.2.2]octane, chloride (SR140333), abrogated these stimulatory effects. In addition, the NEP/CD10 inhibitor stimulated interleukin (IL)-2 production, IL-2 receptor α chain expression, and concanavalin A-induced proliferation of CD5+ thymocytes, and it inhibited spontaneous and NK,R-dependent thymocyte apoptosis. The thiorphan-protective antiapoptotic and proliferative effects involved the activation of Akt serine-threonine kinase, subsequent up-regulation of survivin mRNA, down-regulation of procaspase-3 mRNA levels, and suppression of caspase-3 activity, which were inhibited by SR140333 and mimicked by exogenous SP administration. Overall, our findings suggest that by controlling SP availability, NEP/CD10 negatively regulates thymocyte homeostasis and development.

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Abbreviations:
NEP, neutral endopeptidase; ET-1, endothelin-1; TMPO, thymopoietin; SP, substance P; CGRP, calcitonin gene-related peptide; NK,R, neurokinin-1 receptor; PPT-A, preprotachykinin A; DP, double positive; SR140333, (S)1-{2-[3(3-4-dichlorophenyl)-1-(3-iso-propoxyphenylacetyl)piperidine-3-yl]ethyl}-4-pheny-1-azoniabicyclo[2.2.2]octane, chloride; PI3-K, phosphatidylinositol-3 kinase; IAP, inhibitor of apoptosis; FACS, fluorescent-activated cell sorting; mAbs, monoclonal antibodies; PE, phycoerythrin; FITC, fluorescein isothiocyanate; RAG, rabbit anti-goat; GAR, goat anti-rabbit; HRP, horseradish peroxidase; Ab, antibody; PAGE, polyacrylamide gel electrophoresis; ConA, concanavalin A; [3H]TdR, tritiated thymidine; IL, interleukin; ELISA, enzyme-linked immunosorbent assay; AnnV, annexin V; PI, propidium iodine; PCR, polymerase chain reaction; ANOVA, analysis of variance; RT, real time; IL-2R, IL-2 receptor.
Thus, NEP/CD10 degrades SP by hydrolysis of the Gln6-related peptide (CGRP), and substance P (SP) (Shipp and augmented allergic contact dermatitis, which is abrogated by intestinal inflammation (Lu et al., 1995), and they show highly sensitive to bacterial endotoxins, endotoxic shock, and mRNA, mature SP, and its NK1Rb yC D 4 al., 2002). In this regard, we have provided evidence for the sized and spontaneously released by thymocytes (Santoni et microenvironment (Jurjus et al., 1998), but it is also synthe- sp in the rat thymus not only derives from sensory nerve fiber-mediated axonal transport and release in the thymic microenvironment (Jurjus et al., 1998), but it is also synthe- sized and spontaneously released by thymocytes (Santoni et al., 2002). In this regard, we have provided evidence for the expression of the SP precursor preprotachykinin-A (PPT-A) mRNA, mature SP, and its NK1Rb by CD4+ and CD4+CD8+ (double positive; DP) rat thymocytes.

The involvement of NEP in terminating the proinflamma- tory and immunomodulatory effects of SP has been studied by using selective inhibitors and NEP knockout mice. Highly specific NEP inhibitors, such as thiorphan and phosphoram- idon, have been used as pharmacological tools, and they were found to potentiate the activity of SP (Roques et al., 1980; Matsas et al., 1984; Okamoto et al., 1994). NEP–/– mice are highly sensitive to bacterial endotoxins, endotoxic shock, and intestinal inflammation (Lu et al., 1995), and they show augmented allergic contact dermatitis, which is abrogated by treatment with the NK1R antagonist SR140333 (Scholzen et al., 2001). In addition, these mice showed subtle differences in lymphoid development (Lu et al., 1995). SP can also mod- ulate thymocyte survival pathways (Santoni et al., 2002), although the signaling pathways by which SP affects cell survival are still poorly elucidated.

Akt/protein kinase B is a serine-threonine kinase that me- diates cell survival in various cell types including thymocytes (Coffer et al., 1998); growth factors, cytokines, and hormones cause phosphatidylinositol-3 kinase (PI3-K) activation and generate the membrane phospholipid phosphatidylinositol-3,4,5-trisphosphate that recruits Akt to the membrane, where it becomes phosphorylated at Thr308 and Ser473 res- idues. Once activated, Akt phosphorylates survival-mediated targets, including Bcl-2 family members and caspases, thus inhibiting apoptosis and promoting cell survival (Yang et al., 2004).

Survivin is the smallest member of the recently described inhibitor of apoptosis (IAP) gene family involved in the reg- ulation of mitosis and cell death (Ambrosini et al., 1998). Like other IAP family members, survivin inhibits processing of procaspase-3 and procaspase-7, and it specifically binds the active form of both caspases through a baculovirus IAP repeat domain. The aim of this study was to evaluate the expression of NEP/CD10 on distinct subsets of rat thymocytes at the mRNA and protein levels and to assess its in- volvement on thymocyte survival and proliferation by the analysis of the Akt/survivin pathway and caspase-3 activity.

Materials and Methods

Animals. Specific pathogen-free male Wistar rats (Charles River Italica, Calco, Italy) were used for all experiments. Newborn rats were housed at a constant temperature and humidity with a 12-h light/dark cycle, and they were given food pellets and tap water ad libitum. Age-matched rats were acclimatized before distribution into the experimental groups. No changes on heart rate were found in thiorphan-treated rats compared with untreated and vehicle-treated rats (data not shown). All procedures were done in accordance with the European Communities Council Directive and the guidelines set forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996).

Thiorphan, SP, and NK,R Antagonist (SR140333) Adminis- tration. Thiorphan N-[(RS)-2-benzyl-3-mercaptoanopropanoyl]glycine (Sigma-Aldrich, St. Louis, MO), a specific neutral peptidease inhibitor (Roques et al., 1980) diluted in 10% ethanol, was continuously administered for 7 days alone (2 mg/kg) or in combination with the NK,R antagonist SR140333 (kindly provided by sanofi-aventis, Bridgewater, CT) (Edmonds-Alt et al., 1993) by means of minios- motic pumps (Alza, Palo Alto, CA) subcutaneously implanted in normal rats at day 21 after birth as described previously (Santoni et al., 1995). The starting thiorphan concentration in the pump cham- ber was 7.3 × 10−3 M, with a mean pumping rate of 1.06 ± 0.03 μl/h.

SR140333, diluted in distilled water, was administered alone (20 mg/ml) or in combination with thiorphan as described previously (Santoni et al., 1999). SP (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met) (1 mg/ml), 98.6% pure as assessed by high-performance liquid chromatography (Peninsula Laboratories, Belmont, CA), was diluted in distilled water and administered as described previously (Santoni et al., 1999). As the control group, normal rats received implants filled with the respective vehicles. Five animals from each group were sacrificed at day 28 after birth. Because no major differ- ences were found between the untreated versus thiorphan, SP, or SR140333 vehicle in all of the experiments, for sake the simplicity we use the term vehicle to refer to all of the vehicles used.

We chose to deliver SP, SR140333, and thiorphan by a continuous route of administration, because this modality closely mimics the pathophysiological state observed in immune-mediated diseases, wherein sensory nerves and cells are presumably continuously stim- ulated to release tachykinins, such as SP (Maa et al., 2000).

Whole blood was collected for measurement of the plasma basal levels of corticosterone. Plasma corticosterone levels were deter- mined by radioimmunoassay (MP Biomedicals, Irvine, CA). Cortico- sterone concentrations are expressed as nanogram per milliliter of plasma. The sensitivity was 12.5 ng/ml plasma. Thiorphan, admin- istered as described above, did not affect basal plasma levels of corticosterone, compared with the control groups (thiorphan-treated rats, 20.1 ± 1.5 ng/ml; untreated rats, 19.3 ± 2.0 ng/ml; vehicle- treated rats, 20.5 ± 1.7 ng/ml).

Thymus Cell Preparation. Thymi from untreated rats or rats given thiorphan, SR140333, thiorphan plus SR140333, SP, or their respective vehicles were teased, and cellular debris were removed by intense washing. Thymocytes were isolated by centrifugation on Ficoll-Hyphaque (Lympohrep; Nycomed, Oslo, Norway) gradients, washed twice in RPMI 1640 medium (Flow Laboratories, Irvine, Scotland, UK), and counted and diluted at appropriate concentra- tions in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (Euroclone, Devon, UK), 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg of streptomycin. Cell purity, as assessed by immunofluorescence and fluorescent-activated cell sorting (FACS)
analysis using mouse anti-rat CD5 (monoclonal antibodies; mAbs), was routinely 99%.

**Antibodies.** We used the following mouse mAbs: phycoerythrin (PE)-conjugated and purified anti-rat CD5 (clone OX19), fluorescein-isothiocyanate (FITC)-conjugated anti-rat CD25 (clone OX39), PE-conjugated anti-rat CD8α (clone OX8), and FITC-conjugated anti-rat CD4 (clone W3/25) (obtained from BD Pharmingen, San Diego, CA) and anti-rat α-tubulin (Millipore Bioscience Research Reagents,Temecula, CA). The following affinity-purified polyclonal antibodies were used: goat anti-rat SP raised against the mature peptide, goat anti-rat NR, rabbit anti-rat NEP/CD10, rabbit anti-rat phospho-Akt (Ser473) (Cell Signaling Technology Inc., Danvers, MA), and rabbit anti-rat caspase-3 (Calbiochem-Novabiochem, San Diego, CA). The FITC-conjugated rabbit anti-goat (RAG), FITC-conjugated goat anti-rabbit (GAR), and horseradish peroxidase (HRP)-conjugated RAG were purchased from Biomeda (Foster City, CA). The HRP-conjugated donkey anti-rabbit and RAG-biotinylated and tricolor-conjugated streptavidin were obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK), and the HRP-conjugated sheep anti-mouse was obtained from Invitrogen (Carlsbad, CA). PE-mouse IgG1, FITC-mouse IgG1 (BD Biosciences), as well as goat or rabbit serum (Cappel Laboratories, Durham, NC), were used as negative controls.

**Immunofluorescence and Flow Cytometric Analysis.** To determine the expression of NEP/CD10 on rat thymocytes (99% CD5+ pure), 1 x 10⁶ cells from untreated, vehicle-, thiorphan-, SR140333-, or thiorphan plus SR140333-administered rats were stained with the rabbit anti-rat NEP/CD10 polyclonal antibody (Ab). Normal rabbit serum was used as a negative control. After 30 min at 4°C, the cells were washed twice and labeled with FITC-conjugated GAR (1:20 dilution). The expression of NEP/CD10 on distinct rat thymocyte subpopulations was evaluated by tricolor immunofluorescence and flow-cytometric analysis using PE-conjugated anti-rat CD8α, FITC-conjugated anti-rat CD4, and rabbit anti-rat CD10. In brief, 1 x 10⁶ thymocytes from untreated, vehicle-, thiorphan-, SR140333, or thiorphan plus SR140333-administered rats were labeled with PE-conjugated anti-rat CD8α, FITC-conjugated anti-rat CD4, and rabbit anti-CD10 followed by biotin-conjugated GAR IgG (1:50 dilution) and tricolor-conjugated streptavidin. PE mouse IgG1 and FITC mouse IgG2a were used as negative controls.

To analyze the expression of NK/R and membrane-bound SP on thymocytes, highly purified CD5+ cells from untreated, vehicle-, thiorphan-, SR140333, or thiorphan plus SR140333-administered rats were stained with the rabbit anti-rat SP/CD10 polyclonal antibody (Ab). Normal rabbit serum was used as a negative control. After 30 min at 4°C, the cells were washed twice and labeled with FITC-conjugated GAR (1:20 dilution). The expression of NEP/CD10 on distinct rat thymocyte subpopulations was evaluated by tricolor immunofluorescence and flow-cytometric analysis using PE-conjugated anti-rat CD8α, FITC-conjugated anti-rat CD4, and rabbit anti-rat CD10. In brief, 1 x 10⁶ thymocytes from untreated, vehicle-, thiorphan-, SR140333, or thiorphan plus SR140333-administered rats were double labeled with FITC-conjugated anti-CD5 and PE-conjugated anti-CD25 for 5 min at 4°C and then washed twice. Thymocytes were then analyzed for relative fluorescence intensity. For tricolor cytotoxicometric analysis, electronic compensation was used between green and orange and between orange and red fluorescence to remove spectral overlap. The percentage of positive cells was determined over 10,000 events, and samples were analyzed on a FACSscan cytometer (BD Biosciences, San Jose, CA) by using CellQuest software (BD Biosciences). Fluorescent intensity was expressed as arbitrary units on a logarithmic scale.

**Western Blot.** Lysates that were obtained from untreated thymocytes were resuspended in 0.2 ml of radioimmunoprecipitation assay (0.1% Nonidet P-40, 1 mM CaCl₂, 1 mM MgCl₂, 0.1% sodium azide, 1 mM phenylmethylsulfonyl fluoride, 0.03 mg/ml aprotinin, and 1 mM NaVO₄). Samples were separated on 7% SDS-polyacrylamide gel, transferred to Immobilon-P membranes (Millipore Corporation, Billerica, MA), and blotted with rabbit anti-CD10/NEP/CD10 (1:400 dilution), and then incubated with the HRP-conjugated anti-rabbit (1:10000) Ab. For Akt detection, lysates from untreated, vehicle-, thiorphan-, SR140333-, thiorphan plus SR140333-, or SP-administered rats were resuspended for 15 min in serum-free RPMI 1640 medium, lysed, resolved by 8% SDS-polyacrylamide gel electrophoresis (PAGE), and transferred. Membranes were immunoblotted with anti-Akt Ab (1:500) and anti-phospho-Ser473-Akt Ab (1:1000) followed by incubation with the HRP-conjugated anti-rabbit (1:10000) Ab. For caspase-3 activation, proteins were separated on 14% SDS-polyacrylamide gels, transferred overnight at 20 V, and incubated for 2 h at room temperature with a rabbit anti-caspase-3 Ab (1:500 dilution) followed by donkey HRP-conjugated anti-rabbit (1:10000 dilution) Ab.

Immunoreactivity and densitometric analysis were detected with enhanced chemiluminescence (ECL; Amersham Life Sciences) and analyzed by ChemiDoc and QuantityOne software (Bio-Rad, Hercules, CA). All experiments were performed at least twice using different cell lysates with similar results. Each sample was compared with its control (α-tubulin) for the purpose of quantification.

**Proliferation Assay.** A total of 2 x 10⁵ thymocytes from untreated, vehicle-, thiorphan-, SR140333-, or thiorphan plus SR140333-administered rats were dispersed into tissue culture-treated plastic, 96-well, round-bottom microtiter plates (Corning Life Sciences, Lowell, MA) and cultured for 72 h with 5.0 µg/ml concanavalin A (ConA; Sigma-Aldrich) in a total volume of 200 µl of culture medium at 37°C in a 5% humidified atmosphere. Cell proliferation was evaluated by pulsing the cells with 0.5 µCi/well tritiated thymidine ([³H]Tdr) (6.7 Ci/mmol; PerkinElmer Life and Analytical Sciences, Waltham, MA) during the last 6 h of the 72-h culture. Incorporation of [³H]Tdr was measured by a standard liquid scintillation counting technique after harvesting the cells with the Dynatech harvester (International PBL, Milan, Italy). All cultures were run at least in quadruplicate. Data are expressed as stimulation index and calculated as follows: [³H]Tdr uptake in ConA-stimulated cells/unstimulated cells.

**Interleukin-2 Assay.** Interleukin (IL)-2 levels were measured in the supernatants of unstimulated or 24-h ConA (5 µg/ml) stimulated thymocyte cultures from untreated, vehicle-, thiorphan-, SR140333-, or thiorphan plus SR140333-administered rats by a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN). Results are expressed in picograms per milliliter as the mean concentration ± S.D. of culture supernatants measured in duplicate, and data were pooled from three separate experiments.

**Apoptosis Assays.** Apoptosis of thymocytes from untreated, vehicle-, thiorphan-, SR140333-, or thiorphan plus SR140333-administered rats was evaluated by biparametric flow cytometric analysis. In brief, 10⁶ thymocytes were resuspended in 0.2 ml of binding buffer (10 mM HEPES/NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, and 1.8 mM CaCl₂) in the presence of 5 µl of FITC-Annexin V (‘Ann V’) (Bender MedSystem, Vienna, Austria) and then incubated for 10 min at room temperature in the dark. Cells were washed, resuspended in 0.2 ml of binding buffer containing 10 µl of propidium iodine (PD) (20 µg/ml in phosphate-buffered saline) (Invitrogen) to discriminate apoptotic from necrotic cells, and then analyzed on a FACScan cytometer using the CellQuest software.

**RNA Isolation and Reverse Transcription.** mRNA was extracted by Picoll-purified thymocytes from untreated, vehicle-, SR140333-, thiorphan plus SR140333-, and thiorphan-administered rats and from rat brain tissues (frontal cortex) used as a positive control (Pollard et al., 1989), using the Quick Messenger RNA Direct Kit (Talent, Trieste, Italy). The mRNA samples were resuspended in diethyl pyrocarbonate water (Sigma-Aldrich, Milano, Italy), and their concentration and purity were evaluated by A₂₆₀ measurement. mRNA samples (200 µg) were subjected to reverse transcription using the High-Capacity cDNA Archive Kit (Roche Diagnostics, Mannheim, Italy). The cDNA products were used as template for polymerase chain reaction (PCR) analysis.

**Qualitative and Quantitative Real-Time PCR Analysis.** Qualitative PCR was performed using a GeneAmp 5700 Sequence Detection System (Applied Biosystems). The following forward and
reverse primers specific for rat NEP were used: 5'-CCCCGCCG- GCATTT-3' and 5'-CCCCCATAAGTTCAAGGTTG-3'. PCR products were analyzed by electrophoresis in 2% ethidium bromide-stained agarose gel visualized by UV transilluminator and acquired by using ChemiDoc.

Real-time (RT) PCR was performed using an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). The reaction mixture contained the TaqMan Universal PCR Master Mix (Applied Biosystems) and TaqMan primers and probe sets. TaqMan forward and reverse primers and probe sets were designed from sequences in the GenBank database by Primer Express (Applied Biosystems). PPT-A, NK1R, CGRP, ET-1, TMPO, procaspase-3, and survivin mRNA levels were expressed as relative -fold of that of the corresponding control as indicated in the figure legends. Statistical analysis was carried out on all of the TaqMan PCR data.

Statistical Analysis. The statistical significance was determined by using analysis of variance (ANOVA) followed by post hoc Newman-Keuls multiple comparison at p < 0.01 (+). Results

Expression of CD10/NEP on Rat Thymocytes. Previous findings showed the presence of NEP/CD10 on unfragmented human thymocytes (Mari et al., 1994; Guérin et al., 1997), but direct evidence for the expression of CD10/NEP on distinct thymocyte subset is still lacking.

We initially evaluated the expression of NEP/CD10 mRNA on Ficol gradient-purified (>99% CD5+ ) rat thymocytes and brain tissue used as positive control for the PCR analysis (Pollard et al., 1989). A PCR product of expected size was identified in both rat thymocytes and brain tissue (Fig. 1A).

Expression of NEP/CD10 was then assessed at the protein level. Western blot analysis of thymocyte lysates revealed a single band with an apparent molecular mass of 100 kDa probably corresponding to NEP/CD10, because a similar band was evident in lysates of rat brain tissue (Fig. 1B). No reactivity was observed with normal rabbit serum used as negative control (data not shown). Immunofluorescence and FACS analysis revealed that NEP/CD10 protein is expressed on CD5+ thymocytes (Fig. 1C), mainly on the CD4+CD8- (DP) subset (Fig. 1D).

Thiorphan Administration Up-Regulates Both PPT-A mRNA and SP Protein Expression on Rat Thymocytes. Recent evidence indicates that SP modulates PPT-A mRNA and

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**Fig. 1.** Expression of CD10/NEP mRNA and protein on distinct rat thymocyte subsets. A, NEP/CD10 mRNA levels on rat thymocytes (>99% CD5+ cells) and brain tissue were evaluated by PCR assay. Data shown are representative of three separate experiments. B, lysates from thymocytes and brain tissue were separated on 7% SDS-PAGE and probed with a rabbit anti-rat NEP/CD10 polyclonal Ab. Sizes are shown in kDa, and the arrowhead indicates the band corresponding to NEP/CD10. The data shown are representative of three separate experiments. C, the expression of NEP/CD10 on thymocytes was evaluated by immunofluorescence and FACS analysis using a rabbit anti-rat CD10/NEP polyclonal Ab. Normal rabbit serum was used as a negative control, and goat FITC-conjugated anti-rabbit was used as the secondary Ab. The data are representative of three separate experiments. D, the gray area represents the relative cell number of NEP/CD10-positive cells. Thymocytes were stained with FITC-conjugated anti-rat CD4, PE-conjugated anti-rat CD8 mAb, and rabbit anti-rat NEP/CD10 polyclonal Ab, followed by biotin GAR IgG and tri-color-conjugated streptavidin. The expression of CD4 and CD8 antigens was analyzed by flow cytometry on R1-gated CD10+ rat thymocytes. Data are representative of three separate experiments. Numbers in the cytogram represent the percentage of positive cells.
SP levels in an autocrine fashion upon interaction with its receptor NK1R (Bae et al., 2002; Santoni et al., 2002). Thus, to assess whether inhibition of SP degradation by in vivo thiorphan administration could affect SP expression on CD5⁺ rat thymocytes, we determined both the expression of SP precursor PPT-A at the mRNA level and SP protein levels. As shown by quantitative RT-PCR analysis, a marked increase (approximately 2-fold) in PPT-A mRNA levels was observed on thymocytes from thiorphan-administered rats compared with untreated or vehicle-treated rats (Fig. 2A). No major differences were found by comparing PPT-A mRNA level in thymocytes from vehicle-administered versus untreated rats.

Previous reports indicate the in vivo presence of membrane-bound SP on lymphocytes (Qian et al., 2001). Thus, we evaluated the surface SP immunoreactivity by immunofluorescence and FACS analysis. As indicated in Fig. 2B, thymocytes from thiorphan-administered rats show significantly increased (4-fold) levels of SP (Fig. 2B). This increase is inhibited after concomitant administration of the NK1R antagonist SR140333, thus suggesting that it is mediated by ligand-dependent receptor engagement.

We also analyzed the mRNA levels of other peptide mediators that are NEP substrates, namely CGRP, ET-1, and TMPO, and we found that thiorphan treatment resulted in a significant increase of TMPO levels only (Fig. 3). Taken to-
whether thiorphan administration could modulate NK1R expression in vitro in a ligand-dependent manner. Thus, we analyzed whether thiorphan administration could modulate NK1R expression on thymocytes at mRNA and protein levels.

RT-PCR analysis on total RNA of thymocytes from thiorphan-administered rats showed a 4-fold increase in NK1R mRNA levels, whereas no significant differences were observed on thymocytes from vehicle-administered versus untreated rats (Fig. 4A). We further evaluated the expression of NK1R by immunofluorescence and FACS analysis on CD5+ thymocytes from thiorphan-administered rats treated or not with SR140333; thiorphan administration induced a 3-fold increase of NK1R expression, which was completely inhibited by SR140333 (Fig. 4B). Overall, these results indicate that NK1R thymocytes expression is up-regulated after its activation by endogenous SP.

**Effect of Thiorphan Administration on Total CD10+ Thymocyte Number and Subset Distribution.** Exogenous SP administration has been reported to increase thymocyte numbers (Santoni et al., 2002). Thus, to investigate the functional role of endogenous SP on thymocytes expressing the NEP/CD10 molecule, we initially investigated the ability of thiorphan to affect thymocyte number and subset distribution. NEP/CD10 is expressed on 45.5% of total thymocytes on both DP and CD4+ cells. We found that thiorphan enhances total thymocyte number, and this increase was completely reversed by simultaneous SR140333 administration (Table 1). Cytofluorimetric analysis also showed that thiorphan administration markedly increased the percentage and absolute number of CD10+ CD4+ and CD10+ DP thymocytes; in contrast, SR140333 that was administered simultaneously with thiorphan reverted the thiorphan-induced stimulatory effect (Table 1).

**Thiorphan Administration Induces IL-2 Production, IL-2 Receptor α (CD25) Chain Expression, and Stimulates ConA-Induced Thymocyte Proliferation.** We have previously demonstrated that exogenous SP administration induces CD25 expression, IL-2 production, and stimulates ConA-induced thymocyte proliferation (Santoni et al., 2002). Thus, to investigate the involvement of the IL-2/IL-2 receptor (IL-2R) pathway in the endogenous SP-induced stimulation of thymocyte proliferation, we analyzed the ability of in vivo thiorphan administration to modulate IL-2 and CD25 expression. By ELISA assay, we found that IL-2 is already present in the culture supernatants of unstimulated thymocytes from thiorphan-administered rats and further increases in response to ConA (Fig. 5A). In addition, SR140333 that was simultaneously administered with thiorphan inhibited the endogenous SP-induced IL-2 production; moreover, FACS analysis revealed that thiorphan administration enhances (from 10 to 68%), and SR140333 markedly reduces (from 68 to 24%), basal CD25 expression (Fig. 5B).

Finally, we analyzed whether increased levels of endogenous SP by means of thiorphan administration could affect thymocyte proliferation. Evaluation of ConA-induced proliferative response of thymocytes from untreated rats or rats given vehicle, thiorphan, SR140333, and thiorphan plus SR140333 demonstrates that thiorphan stimulates ConA-induced thymocyte proliferation (Fig. 5C). Thymocyte proliferation is SP-mediated and NK1R-dependent as shown by the ability of the NK1R antagonist to completely revert the thiorphan-induced effects (Fig. 5C). As previously demonstrated (Santoni et al., 2002), SR140333 alone slightly inhibited IL-2-mediated thymocyte responses (Fig. 5, A–C). Taken together, these results show that thiorphan exerts a stimulatory effect on ConA-induced thymocyte proliferation through increased levels of endogenous SP.

**Thiorphan Administration Rescues Rat Thymocytes from Spontaneous and NK1R Antagonist-Induced Apoptosis.** We have previously demonstrated that exogenous SP administration rescues thymocytes from spontaneous and NK1R antagonist-induced apoptosis (Santoni et al., 2002). Thus, we investigated, by biparametric flow cytomet-
Effect of thiorphan administration on total rat thymocyte number and CD10+ thymocyte subset distribution

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell Number × 10^8/Thymus</th>
<th>%CD10+</th>
<th>%CD10+DP</th>
<th>%CD10+CD4*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>7.5 ± 0.4</td>
<td>45.5 ± 1.9</td>
<td>41.6 ± 0.9</td>
<td>3.9 ± 0.18</td>
</tr>
<tr>
<td>Vehicle</td>
<td>8.2 ± 0.5</td>
<td>43.8 ± 2.5</td>
<td>39.2 ± 0.8</td>
<td>4.4 ± 0.24</td>
</tr>
<tr>
<td>Thiorphan</td>
<td>12.3 ± 0.8**</td>
<td>65.3 ± 2.8**</td>
<td>57.6 ± 0.8**</td>
<td>7.7 ± 0.26**</td>
</tr>
<tr>
<td>SR140333</td>
<td>6.4 ± 0.4**</td>
<td>38.7 ± 0.4**</td>
<td>35.8 ± 0.6**</td>
<td>2.9 ± 0.06**</td>
</tr>
<tr>
<td>Thiorphan + SR140333</td>
<td>7.2 ± 0.9**</td>
<td>43.7 ± 1.9**</td>
<td>40.3 ± 1.9**</td>
<td>3.4 ± 0.09**</td>
</tr>
</tbody>
</table>

* p < 0.01 was determined by ANOVA, comparing thiorphan-administered rats with untreated, vehicle- and SR140333-administered rats and SR140333-administered rats with untreated, vehicle-, or thiorphan plus SR140333-administered rats.

Thiorphan Mediates Survival/Proliferation of Rat Thymocytes

**Thiorphan Administration Induces Akt Phosphorylation on Rat Thymocytes.** To investigate the signaling pathway underlying the SP-mediated thymocyte rescue from apoptosis, we examined whether thiorphan administration could result in changes of Akt phosphorylation, a signaling event controlling cell survival (Coffer et al., 1998). Western blotting using an antibody to Ser473-phosphospecific Akt revealed increased (3-fold) Akt phosphorylation on rat thymocytes upon thiorphan administration (Fig. 6). In contrast, the SR140333 administered simultaneously with thiorphan markedly inhibited thiorphan-induced Akt phosphorylation. Like the NEP inhibitor, SP administration stimulated Akt phosphorylation, further supporting the fact that thiorphan-induced Akt phosphorylation is mediated by increased levels of endogenous SP.

**Thiorphan Administration Up-Regulates Survivin and Reduces Procaspase-3 mRNA Level on Rat Thymocytes.** The mechanisms by which Akt promotes survival involve the antiapoptotic inhibitor survivin (Ohashi et al., 2004), which inhibits executive caspase activity (Dan et al., 2004). Thus, we examined whether thiorphan administration could modulate survivin and procaspase-3 mRNA expression. We found that thiorphan administration induces a marked increase (2-fold) of survivin mRNA levels, which were significantly inhibited by SR140333 (Fig. 7A); similarly, enhanced survivin mRNA expression was observed after SP administration.

Furthermore, thiorphan treatment was accompanied by a marked down-regulation of procaspase-3 mRNA levels on thymocytes. This inhibitory effect was completely reverted by SR140333 administration (Fig. 7B) and mimicked by exogenous SP. No major differences on procaspase-3 and survivin mRNA expression were observed upon SR140333 administration. Overall, these results suggest that thiorphan in vivo induces SP-dependent and NK1R-mediated thymocyte sur-
vival both by inhibiting procaspase-3 and enhancing survivin gene expression.

**Thiorphan Administration Reverts Caspase-3 Activation on Rat Thymocytes.** Constitutive caspase-3 activity has been described in mouse DP thymocytes (Jiang et al., 1999), and recently the ability of survivin to interact with, and to inhibit, caspase-3 activity has been demonstrated (Ohashi et al., 2004). Thus, we analyzed the state of caspase-3 activation in rat thymocytes from untreated, vehicle-, thiorphan-, SR140333-, or thiorphan plus SR140333-administered rats. We found that caspase-3 is constitutively activated in untreated thymocytes, as demonstrated by the appearance of the 17-kDa fragment of caspase-3 (Fig. 8). Thiorphan administration significantly inhibited caspase-3 activation in an NK1R-dependent manner, as shown by the ability of SR140333 administration to completely revert the thiorphan-mediated effects. Likewise, exogenous SP inhibited the basal level of caspase-3 activation. No major differences on caspase-3 activation were observed in thymocytes from vehicle-treated versus untreated rats. Overall, these results suggest that thiorphan in vivo induces SP-dependent and NK1R-mediated inhibition of caspase-3 activation on rat thymocytes.

**Discussion**

The local bioactivity of SP in the rat thymus seems to be tightly controlled by its synthesis/release from sensory nerves (Jurjus et al., 1998) and thymocytes (Santoni et al.,

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**TABLE 2**

Effect of thiorphan administration on total rat thymocyte number and CD10⁺ thymocyte subset distribution

<table>
<thead>
<tr>
<th>Group</th>
<th>PI⁺ AnnV⁺</th>
<th>PI⁺ AnnV⁻</th>
<th>PI⁻ AnnV⁺</th>
<th>PI⁻ AnnV⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>78.3 ± 1.9</td>
<td>0.5 ± 0.1</td>
<td>2.9 ± 0.3</td>
<td>18.3 ± 1.3</td>
</tr>
<tr>
<td>Vehicle</td>
<td>79.5 ± 1.5</td>
<td>0.4 ± 0.1</td>
<td>4.2 ± 0.6</td>
<td>15.9 ± 1.8</td>
</tr>
<tr>
<td>Thiorphan</td>
<td>91.3 ± 1.8**</td>
<td>0.4 ± 0.1</td>
<td>2.7 ± 0.2</td>
<td>5.6 ± 0.9**</td>
</tr>
<tr>
<td>SR140333</td>
<td>30.6 ± 2.3**</td>
<td>0.5 ± 0.1</td>
<td>36.1 ± 2.2**</td>
<td>32.8 ± 1.9**</td>
</tr>
<tr>
<td>Thiorphan + SR140333</td>
<td>73.9 ± 2.6**</td>
<td>0.4 ± 0.1</td>
<td>7.7 ± 1.0**</td>
<td>18.0 ± 1.7**</td>
</tr>
</tbody>
</table>

**p < 0.01 was determined by ANOVA, comparing thiorphan-administered rats with untreated, vehicle-, SR140333-, or thiorphan plus SR140333-administered rats and SR140333-administered rats with untreated, vehicle-, or thiorphan plus SR140333-administered rats.**

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**Fig. 6.** Thiorphan induces Ser473 Akt phosphorylation on rat thymocytes. Lysates of thymocytes from untreated, vehicle-, thiorphan-, SR140333-, thiorphan plus SR140333-, or SP-administered rats, resuspended in serum-free media, were analyzed for Ser473-phosphorylated Akt by Western blotting using a rabbit anti-rat phospho Akt Ab. The blot was stripped and reprobed with rabbit anti-rat Akt polyclonal Ab. The relative Akt Ser473 phosphorylation levels on thymocytes were determined by densitometric analysis using a ChemiDoc apparatus and Akt protein level as a loading control. Data are represented as mean ± SD of three separate experiments. Statistical analysis was determined by ANOVA; **, p < 0.01 as described above.

**Fig. 7.** Thiorphan modulates survivin and procaspase-3 mRNA expression in rat thymocytes. Survivin (A) and procaspase-3 (B) mRNA level were evaluated by quantitative real-time PCR on thymocytes (>99% CD5⁺ cells) from untreated, vehicle-, thiorphan-, SR140333-, thiorphan plus SR140333-, or SP-administered rats. Results (mean ± S.D.) are normalized for β-actin expression. Survivin and procaspase-3 levels were expressed as relative fold with respect to untreated rats used as controls. Data shown are representative of three separate experiments. **p < 0.01 was determined by ANOVA, comparing thiorphan-administered rats with untreated, vehicle-, SR140333-, or thiorphan plus SR140333-administered rats and SP with untreated, vehicle-, SR140333-, or thiorphan-administered rats.
the NK₃R antagonist SR140333 resulted in down-regulated expression of PPT-A mRNA and SP protein as well as of NK₃R mRNA and protein levels. These findings are consistent with previous evidence indicating that SP itself promotes its own synthesis and NK₃R expression in an NK₃R-dependent manner (Bae et al., 2002; Santoni et al., 2002). In addition, we also observed that thiorphan administration enhanced the thymocyte mRNA levels of TEMPO, but not those of other thymic NEP substrates including ET-1 and CGRP.

Our study also provides evidence that inhibition of NEP/CD10-mediated SP breakdown on rat thymocytes results in enhanced SP-mediated functional responses. A marked increase of total CD10 thymocyte numbers was detected in thiorphan-administered rats, and this increase was specifically inhibited by the SR140333 antagonist. The increased thymocyte number was accompanied by enhanced IL-2 production, increased CD25 expression, and ConA-induced thymocyte proliferation, suggesting that the IL-2/IL-2R system plays a major role in the NEP inhibitor-induced SP-mediated regulation of thymocyte-proliferative response.

The evidence that the NK₃R antagonist did not completely reverse thiorphan-mediated increase of CD25 expression and IL-2 release, together with the observation that thiorphan administration enhances TEMPO mRNA expression, also suggest the involvement of TEMPO NEP substrate in the control of these functional responses. In this regard, TEMPO has been described to stimulate IL-2 release in T cells (Guérin et al., 1997).

In addition, in accordance with previous evidence showing that NEP/CD10 regulates cell survival (Shipp and Look, 1993; Sumimoto et al., 2001), and that exogenous SP protects thymocytes from spontaneous and NK₃R antagonist-induced apoptosis (Santoni et al., 2002), we found that CD10/NEP activity regulates the expression and activation of an important component of antiapoptotic responses, namely the Akt/survivin pathway, by controlling SP levels.

Akt activation is a critical signaling event that controls survival of a variety of cell types, including thymocytes (Jones et al., 2000; Kelly et al., 2002), and overexpression of activated Akt was reported to selectively regulate survival of murine DP and SP thymocytes (Jones et al., 2000). In addition, a recent report describes the ability of SP to stimulate Akt phosphorylation and antiapoptotic responses on human colonocytes (Koon et al., 2007).

In this regard, we found that thiorphan administration increased Ser473 Akt phosphorylation on rat thymocytes in an NK₃R-dependent manner, and that coadministration of SR140333 antagonist and thiorphan completely reverted the NEP-mediated SP stimulation of Akt phosphorylation; moreover, we observed that administration of SP itself stimulates Akt phosphorylation.

We also showed that thymocytes from thiorphan- or SP-treated rats exhibited increased mRNA expression of survivin, an antiapoptotic inhibitor involved in the Akt promotion of cell survival (Ambrosini et al., 1998; Dan et al., 2004). The importance of survivin in the regulation of thymocyte survival is also supported by recent evidence describing the fact that loss of survivin blocks the transition from double-negative to DP thymocytes and triggers their growth arrest and cell death (Okada et al., 2004). Like other thiorphan-mediated effects, enhanced survivin mRNA expression was...
also attributable to increased SP bioavailability and activity because it was reverted by simultaneous administration of SR140333.

The antiapoptotic activity of survivin can be mediated by its ability to interact with caspase-3 and to inhibit caspase-3-mediated apoptosis (Ohashi et al., 2004). In this regard, in agreement with previous findings on mouse DP thymocytes (Jiang et al., 1999), we observed that caspase-3 is constitutively activated in untreated thymocytes; thiorphan administration completely inhibited the caspase-3 activation and paralleled increased the basal level of procaspase-3 protein, and this inhibition was reverted by SR140333, thus supporting a role for SP in thiorphan-mediated effects.

Our findings provide the first evidence for a critical in vivo role of NEP/CD10 in the regulation of thymocyte proliferation and survival by controlling the endogenous levels of SP. In addition, they shed light on some of the antiapoptotic and proapoptotic molecular mechanisms, namely activation of the PI3-K/Akt/survivin pathway and inhibition of procaspase-3 expression and caspase-3 activity, underlying SP-mediated promotion of thymocyte survival. Our results are consistent with previous evidence indicating that PI3-K inhibitors down-regulate survivin expression and increase the caspase activity that is associated with cell death (Sommer et al., 2003).

At present, it is unclear whether the in vivo antiapoptotic effects mediated by increased bioavailability of endogenous SP or by exogenous SP administration are directly and/or indirectly related to its ability to stimulate IL-2 release. In this regard, growing evidence indicates that SP exerts direct antiapoptotic effects, and they have also been attributed to its ability to trigger Akt activation (Koon et al., 2007). On the other hand, an important role for IL-2 in regulating T cell apoptosis and survival, activating the PI3-K/Akt pathway (Jones et al., 2000; Kelly et al., 2002), and up-regulating survivin expression has also been reported. Nonetheless, unlike SP, IL-2 has not been implicated in modulation of procaspase-3 expression and/or caspase-3 activity (Sabbagh et al., 2004). This finding, together with the ability of exogenous SP to stimulate Akt phosphorylation and survivin expression, strongly supports the direct role of SP in mediating antiapoptotic responses in rat thymocytes.

What is the pathophysiological role of the SP/NK,R/NEP pathway? The expression of SP, NK-R, and NEP/CD10 on thymocyte subsets strongly supports the hypothesis that continuous SP release in the thymus regulates T cell homeostasis and development by acting as both survival and growth-promoting factors. By controlling the availability of SP in the thymic environment under basal circumstances, NEP/CD10 represents an important negative feedback mechanism that limits SP-immunostimulatory effects. Moreover, increased SP levels have been associated with immune-mediated inflammatory conditions such as arthritis, asthma, allergic contact dermatitis, and inflammatory bowel diseases (Brain and Cox, 2006), and depletion of SP levels by NEP/CD10 has been found to ameliorate these inflammatory responses. A better understanding of the mechanisms by which NEP/CD10 regulates SP bioavailability and function in the thymus will provide novel insights for potential NEP/CD10-based therapeutic intervention in the treatment of these inflammatory and autoimmune disorders.

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References


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