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Mycobacterium smegmatis synthesizes *in vitro* androgens and estrogens from different steroid precursors.

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Abstract

Fast-growing mycobacteria as *Mycobacterium sp and smegmatis* degrade natural sterols. They are a model to study tuberculosis. Interestingly, *Mycobacterium smegmatis* (*M. smegmatis*) have been found in river effluents derived from paper production and therefore it would important to gain further insight in their capacity to synthesize steroids that are potential endocrine disruptors affecting the development and reproduction of fishes. To our knowledge, the capacity of *M. smegmatis* to synthesize estrogens and even testosterone had not been reported. Therefore, the objective of this study was to investigate the capacity of *M. smegmatis* to synthesize *in vitro* testosterone and estrogens from tritiated precursors and to investigate the metabolic pathways involved. Results obtained by thin layer chromatography showed that ^{3}H-progesterone was transformed to 17OH-progesterone, androstenedione, testosterone, estrone and estradiol after 6, 12 or 24 h of incubation. ^{3}H-androstenedione was transformed to testosterone and estrogens mainly estrone, and ^{3}H-testosterone to estrone and androstenedione. Incubation with ^{3}H-dehydroepiandrosterone rendered androstendiol, testosterone and estrogens. The ability of transforming less potent sex steroids as androstenedione and estrone to other more active like testosterone and estradiol or viceversa suggests that *M.smegmatis* influence the amount of self synthesized strong androgens and estrogens and can transform those found in the environment.

Key Words: *Mycobacterium*, *Mycobacterium smegmatis*, Steroid synthesis, Androgens, Estrogens

INTRODUCTION

The mycobacteria (*M*) include a number of human pathogens of worldwide importance, such as *Mycobacterium tuberculosis (MTB)*, *Mycobacterium leprae*, and *Mycobacterium avium*. *ycobacterium tuberculosis* is still a major killer and constitutes a serious menace to global health. A fast growing member of this genus, *Mycobacteriun smeg*matis (*M. smeg*) is another interesting microorganism. Despite the evolutionary differences between *M. smegmatis* and *M*TB, the first one can produce the *M*TB ESAT-6 and CFP-10 proteins, suggesting that substrate recognition is also conserved between the two species. Several authors reported comparative studies of different genes as *whmD eg*, an essential gene in *M. smeg*, involved in cell division while its counterpart present in *MTB, whiB2*, is functionally equivalent. The existence of genes that share significant sequence homology in coding and non-coding DNA in both species *MTB* and *M. smegmatis* had been also demonstrated (Raghunand and Bishai, 2006; Rajagopalan et al. 2005; Goehring and Beckwith, 2005) . Therefore, *M. smegmatis* is considered a strong system to study several products, like the multicomponent Snm secretory machine (a significant determinant of *MTB* virulence) and to appreciate the role of this conserved system in mycobacterial biology (Converse and Cox, 2005).

On the other hand, studies had demonstrated that fast-growing mycobacteria degrade natural sterols and use them as a source of carbon and energy. A number of bacteria have been reported to accumulate some sterols and metabolize them to intermediates such as 4-androstene-3,17-dione and 1,4-androstadiene-3,17-dione (Mahato and Garai, 1997, Martin, 1977, Szentirmai, 1990, Brzostek, et al. 2005,). These intermediates could be useful in industry as precursors for the production of steroid drugs and hormones (Sedlaczek, 1988). The capacity of some microorganisms, represented by *Mycobacterium*, *Corynebacterium* and *Arthrobacter* to make use of sterols as sole sources of carbon and energy, was described years ago by Soehngen (1913).
17β-hydroxysteroid dehydrogenase (17β-HSD) is a crucial enzyme that catalyzes the reversible reduction of 17-keto group of androgens and estrogens in vertebrates and invertebrates. To our knowledge, 14 types of mammalian 17β-HSD have been described or annotated in public databases, but only 12 have orthologues in humans (Lukacik et al. 2006). It has been reported the isolation and characterization of this enzyme in several species of bacteria such as *Alcaligenes sp* (Payne and Talalay, 1985) and *Comamonass testosteroni* (*Pseudomonas testosterone*) (Groman and Engel, 1977), *Cylindrocarpon radicicola* (Itagaki and Iwaya, 1988) as well as fungi (Lanisnik et al. 1999). This enzyme was detected also by differential centrifugation in *Pseudomonas testosterone* as membrane-bounded and cytosol soluble forms (Lefebre et al. 1979). Additionally, two activities of reductive 1-ene-steroid reductase and 17-keto steroid reductase were observed in

Mycobacterium. sp (Goren et al 2002). Two 17OH-HSDs from a mutant *Mycobacterium sp* have been also isolated and purified. One of them is responsible for the bidirectional redox oxygen function at C17, while the other specifically catalyzes the oxidation of 17-beta-steroids such as testosterone and dihydrotestosterone (Egorova et al. 2002 a and b).

Sterol synthesis by the saprofitic microorganism *M.smegmatis* was reported using ^{14}C radiolabelled mevalonic acid and incorporation to into C4-desmethyl sterol co migrating with authentic cholesterol on TLC (Lamb et al, 1998). Microbial transformation of 3-hydroxy-5,6-cyclopropanocholestanes into 17-keto steroids, between them androstenedione (A4), had been reported in *Mycobacterium sp* (Yan et al. 2000) and in *M.smegmatis* (Naghib et al. 2002). Jenkins et al (2004) had demonstrated that *M. smegmatis* incubated in the presence of progesterone produced 17α-hydroxyprogesterone (17α-OHP), androstenedione (A4) and androstadienedione (ADD). Given *M. smegmatis* biological characteristics and for being safety for laboratory use, this strain had been used as a model for many *MTB* studies.

Interestingly, *M. smegmatis* have been found in river effluents derived from paper production (Jenkins et al. 2003; Jenkins et al. 2004) and it has been previously reported the presence of progesterone and androstenedione in the water column and bottom sediments of the Fenholloway River, Taylor County, Florida which receives paper mill effluent and contains masculinized female mosquitofish, *Gambusia Holbrooki*. It was hypothesized that plant sterols (e.g., β-sitosterol) derived from the pulping of pine trees are transformed by bacteria into progesterone and subsequently into 17α-OHP, androstenedione, and androstadienedione (ADD) by degradation of phytosterols (Roy et al. 1991; Durham et al. 2002; Jenkins et al. 2003). In a later study, it was demonstrated that these same androgens can be produced in vitro by *M. smegmatis* (Jenkins 2004). Therefore it would important to further study their capacity to synthesize steroids that may work as endocrine disruptors affecting the development and reproduction of fishes (Segner et al. 2003; Brion et al. 2004; Fenske et al. 2005; Waye and Trudeau, 2011).

To our knowledge, the capacity of *M. smegmatis* to synthesize estrogens and even testosterone had not been reported. Although several authors have described microorganisms capable of degrading cholesterol, sterols and steroids, the routes of microbial transformation/degradation of these compounds, some of which produce aromatic intermediates are not known in detail. Understanding of these routes is of great interest to both the industry and the environment as this would allow manipulation through genetic engineering and new intermediaries for metabolic / synthetic steroid precursors as well as important microorganisms used for purification processes of the water.

Therefore, the objective of the present study was to get deeper insight into the capacity of *M. smegmatis* to synthesize *in vitro* testosterone and estrogens from different precursors, and simultaneously to investigate the metabolic pathways involved in the steroid synthesis or degradation produced by these microorganisms.

Materials and Methods

Mycobacterium smegmatis culture:

Mycobacterium smegmatis PTCC 1307 (CIP 73.26), provided by the National Institute of Respiratories Diseases, Santa Fe, Argentine, Dr. Emilio Coni, was used as a microbial agent, from a culture of *M. smegmatis* developed in Middlebrook 7H10 media (Sigma-Aldrich Chemical Co, St. Louis, MO, USA). The bacteria were seeded in Middlebrook 7H9 liquid medium purchased in the same company.

Mycobacterium smegmatis was grown to mid-log phase (optical density at 600 nm [OD 600], 0.6 to 0.8) in 7H9 media supplemented with 0.05% Tween 80 (Sigma-Aldrich Chemical Co, St. Louis, MO, USA).

Bacilli were grown in this medium for 48h until a concentration of $1.5x10^9$ bacteria / ml was reached. Mycobacteria presence was confirmed by Ziehl-Neelsen stain. To detect any other type of bacterial contamination in liquid culture media containing *M. smegmatis* 100 µL of each of the jars were seeded on a 5% blood agar plate, and incubated for 24h at 28ºC.

Evaluation of mycobacteria concentration

To determine the concentration of mycobacteria the McFarland nephelometer was used. Serial dilutions of the sample were seeded in Middlebroock medium and subsequently counted up of 7H10 plates grown at 28°C was performed, and the OD data (at 600nm) were obtained by the spectrophotometer measures.

Precursor transformation

The bacteria were developed in Middlebrook 7H9 medium for 48h with stirring until a concentration of $1.5x10^9$ bacteria / ml. was reached. The culture was fractionated in 1 ml aliquots. Bacilli were cultured for 6, 12 or 24 h with various tritiated precursors separately in quadruplicate. ^{3}H-Progesterone, ^{3}H-Dehydroespiandrosterone ^{3}H-Androstenedione, ^{3}H-testosterone (^{3}H-P4, ^{3}H-DHEA, ^{3}H-A4 and ^{3}H-T, respectively), at a concentration of 50,000 counts/ml were used as precursors. Simultaneously, tubes containing culture medium without bacteria were incubated with the tritiated precursors (control tubes, blanks). After incubation time, bacteria were separated by centrifugation at 1200 g for 15 min, resuspended in PBS and centrifuged again. The washing

was repeated two times and finally the pellet was frozen until extraction. The bacteria culture media was reserved until steroid extraction was done.

Steroids extraction

The bacteria culture media was poured to glass tubes and steroids were ether-extracted with diethyl ether to a 1:5 ratio, in a bath at 38-40°C. The mixture was stirred by vortex for 1 minute, and left to stand for 10 minutes. The vials were placed in a mixture of dry ice-acetone (-70ºC) for 15 minutes to freeze the aqueous phase and thereafter the ether phase containing the steroids was decanted and dried under N_2 gas. The samples were stored until steroids were submitted to thin layer cromatography (TLC) as described in Valdez et al. (2006).

Thin Layer cromatography

The authentic standards used were progesterone, 17-OH-Progesterone (17OH-P_5), androstenedione (A_4), dehydroespiandrosterone (DHEA), androstenediol (Adiol), testosterone (T_4) , estrone (E_1) and estradiol (E_2) (Steraloids Wilton NH).

The extracted steroids were solubilized in 100 µL of absolute ethanol and 20 µL were seeded in Silica gel 60 F_{254} pre-coated sheet plates (Merck, Darmstadt, Germany) together with the authentic standards. To perform the chromatographic runs the system dichloromethane: ethyl acetate (8:2 v / v) was used.

The standards were detected on the plates by UV light and 10% sulfuric acid followed by heat. The region was used for standard grid plate. Subsequently, the corresponding samples region was cut, and placed in vials with 5 mL of scintillation liquid. Finally, the radioactivity was quantified in a counter for Beta emissions. The results were expressed as percentage transformation of each identified metabolite from the corresponding tritiated precursor.

Statistical analysis

Statistical analysis was performed using Prism version 4. 2003 (GraphPad Software Inc.). Data are presented as means ± SE. Probability values of $P < 0.05$ were considered to be significant. Kruskal-Wallis followed by Dunn´s multiple comparison post-test, were used to investigate statistical differences between groups. The experiments were performed by quatriplicate.

RESULTS

Transformation of triated precursors

Figures 1, 2, 3 and 4 show the graphs which depict the percentage of transformation of the different tritiated precursors to steroid metabolites at 6, 12 or 24 h of incubation (A, B, and C

respectively). Different metabolite percentages of transformation were observed, depending on the precursor from which they proceeded, as well as of the time of incubation.

Figure 1 A show that ^{3}H-Progesterone (^{3}H-P4) was transformed to 17OH-P4, A4, T, E1 and E2 after 6 h of incubation. The same metabolites were found at 12 and 24 h of culture, but their relative proportions changed (Fig.1 B and C). Tritiated testosterone significantly increased along time in culture (6 h vs 24 h $P < 0.05$) while 17OH-P4 decreased (6 h vs 24 h $P< 0.01$). Transformation of the precursors to E1 and E2 was also observed at 12 and 24 h, however their transformation decreased with time in culture (Fig. 1 A and B, 6 h vs 12 h $P< 0.001$).

Incubation of cells with 3H-A4 resulted in transformation to testosterone and to a small percentage of E1 and E2 after 6h of culture (Fig. 2A). After 24 h of culture, testosterone synthesis slightly increased (6 h vs 24 h $P<0.05$), but the most striking change was that of E1 synthesis that reached values similar to those of testosterone in the course of the experiment (6 h vs 12 h $P<0.05$, 12 vs 24 h non significant) while E2 almost disappeared (Fig. 2 B and C).

Tritiated testosterone was transformed to A4, E1 and E2 after six hours of culture (Fig. 3A). Estrone as well as E2 synthesis augmented after 12 h (E1, 6 h vs 24 $P<0.05$; E2, 12 h vs 24 h $P<0.05$) while androstendione decreased at 24 h of culture (6 h vs 24 h $P<0.05$, Fig. 3 B and C).

Figure 4 shows the transformation of tritiated DHEA to its metabolites. The synthesis of Adiol, testosterone and estrogens was found after six hours of culture (Fig. 4A). No significant changes along time of culture were observed in the case of testosterone and estradiol, but Adiol had a trend to increase at 24 h (Fig 4 B and C). The synthesis of estrone was found to be increased when 12 and 24 h were compared ($P<0.05$).

DISCUSSION

In this study we have explored the capacity of *Mycobacterium smegmatis* to transform steroid hormone precursors to sex steroids. The results showed that *M. smegmatis* can use both the Δ4 and the Δ5 steroidogenic pathways to achieve the sex steroid synthesis. The increase of the final products of the pathway and the decrease of the precursors that act as substrates of the reaction suggests the existence of steroidogenic enzymes that are required for these metabolic transformations. Such enzymes would be homologous to the steroidogenic enzymes found in vertebrates and invertebrates, and even in some microbes.

The presence of androgens and estrogens after incubation of *M. smegmatis* with tritiated progesterone showed that this microorganism could synthesize metabolites through the Δ4 steroidogenic pathway. The synthesis of 17α-HO-progesterone, A4 and ADD by *M. smegmatis*

was previously shown by Jenkins et al. (2004). In the present study we found that this microbe also synthesize testosterone and estrogens, suggesting that *M. smegmatis* can aromatize androgens to yield estrogens through the activity of a P-450 aromatase similar to that found in vertebrates and some invertebrates. The estrogen yields found here also raised concern about the presence of estrogens in rivers receiving paper mill effluents.

The estrogen synthesis obtained after incubation of *M. smegmatis* with A4 or T further demonstrate their capacity to synthesize these hormones. Interestingly, incubation in the presence of T resulted in an important transformation to A4 suggesting that a bidirectional pathway is present in *M. smegmatis*. The transformation of T to A4 could be a defense mechanism to degrade potent androgens to compounds with a lesser androgenic capacity. As shown at 12 and 24 h of incubation with tritiated A4, E1 increased while A4 decreased suggesting that the latter steroid was being actively transformed to E1. However transformation of T to E2 and thereafter to E1 is another possibility that should be considered. In general, E1 was the predominant estrogen synthesized by *M. smegmatis* suggesting again a defense mechanism to degrade potent estrogens to compounds with lesser activity.

Androgens and estrogens were also detected in this study when ^{3}H-DHEA was the precursor. The synthesis of A4 from DHEA observed in this study after 6h of incubation requires the activity of a 3β-hydroxysteroid dehydrogenase. The intracellular presence of this enzyme have been described in a mutant strain of *Mycobacterium sp* (Egorova et al. 2005) and an extracellular 3β-hydroxysteroid oxydase form was found in *Mycobacterium vaccae* by Nicolayeva et al. (2004). The synthesis of Adiol found in our study also suggests the presence of a 17β-OH-steroid dehydrogenase like in *M. smegmatis.* Afterward, experiments using DHEA as the precursor strongly suggest that these microorganisms can connect Δ5 and Δ4 steroidogenic pathways.

The ability to transform weak sex steroids as androstenedione and estrone to more active ones like testosterone and estradiol or viceversa, suggests that *M.smegmatis* has the capacity to modulate the amount of strong androgens and estrogens manufactured by themselves and / or those found in the environment.

The results of this investigation strongly suggest the existence in *M.smegmatis* of steroidogenic pathways similar to those already described for vertebrates and some invertebrates, such as Δ4 and Δ5 steroidogenic pathways, in which, starting from cholesterol or other steroid precursors, intermediates are converted in their final metabolites.

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Legends for figures

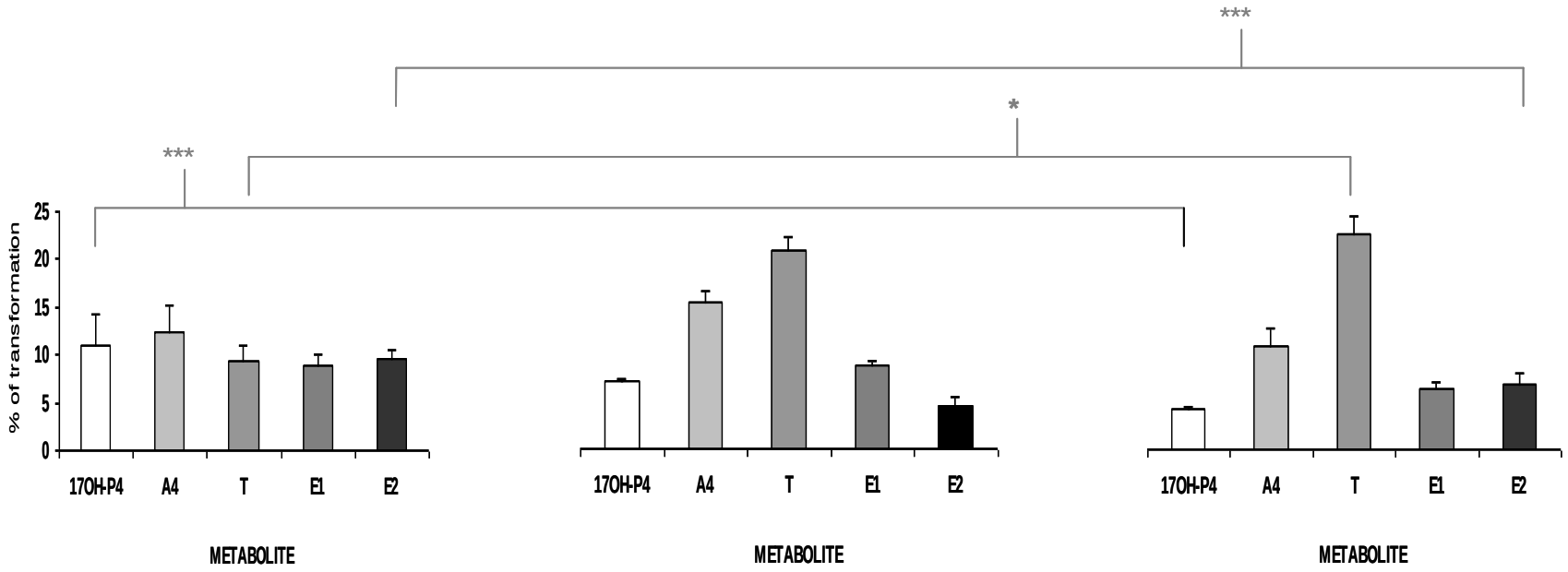
Figure 1. Tranformation of ^{3}H-progesterone to sex steroid metabolites by *M. smeg* after different periods of incubation with the precursor. The bacteria were incubated for 6 (A), 12 (B) or 24 (C) hours in the presence of tritiated progesterone. Data show the mean ± SE

Figure 2. Metabolism of ^{3}H-androstenedione to its metabolites by *M. Smeg*. Tritiated androstenedione, estrone and estradiol were found after 6 (A), 12 (B) or 24 (C) hours of incubation. Data show the mean ± SE.

Figure 3. Transformation of ^{3}H-testosterone to steroid metabolites by *M. Smeg*. The microorganisms were incubated for 6 (A), 12 (B) or 24 (C) hours in the presence of the tritiated precursor. Data show the mean ± SE.

Figure 4. Metabolism of ^{3}H-DHEA to its metabolites by *M. Smeg*. Tritiated androgens and estrogens were found after 6 (A), 12 (B) or 24 (C) hours of incubation with the precursor. Data show the mean ± SE.

Figure 1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% of transformation
25
20
15
10
5
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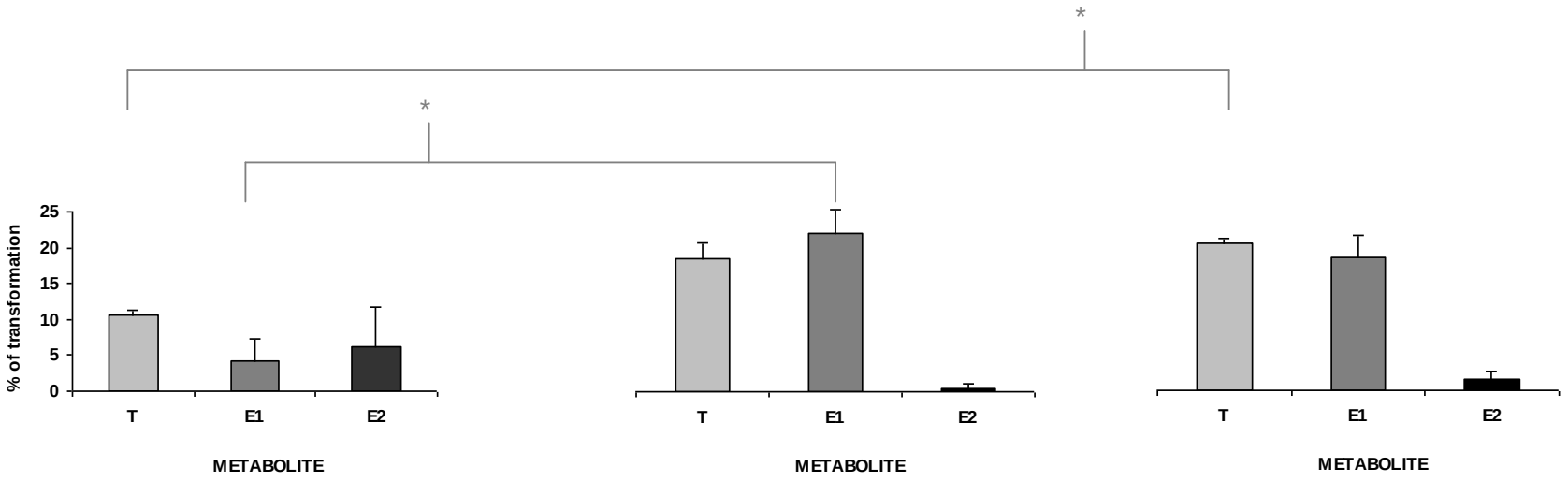
A4
T
E1
E2
METABOLITE
17OH-P4
A4
T
E1
E2
METABOLITE
17OH-P4
A4
T
E1
E2
METABOLITE

(A) (B) (C)

Figure 2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% of transformation
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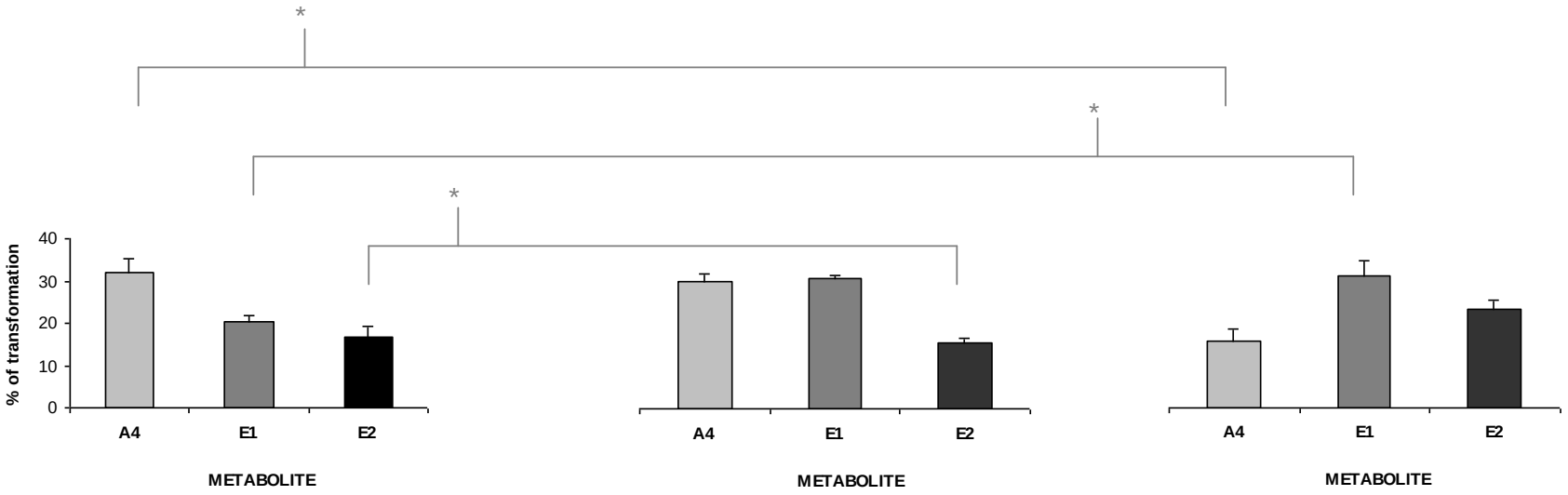
(A) (B) (C)

Figure 3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% of transformation
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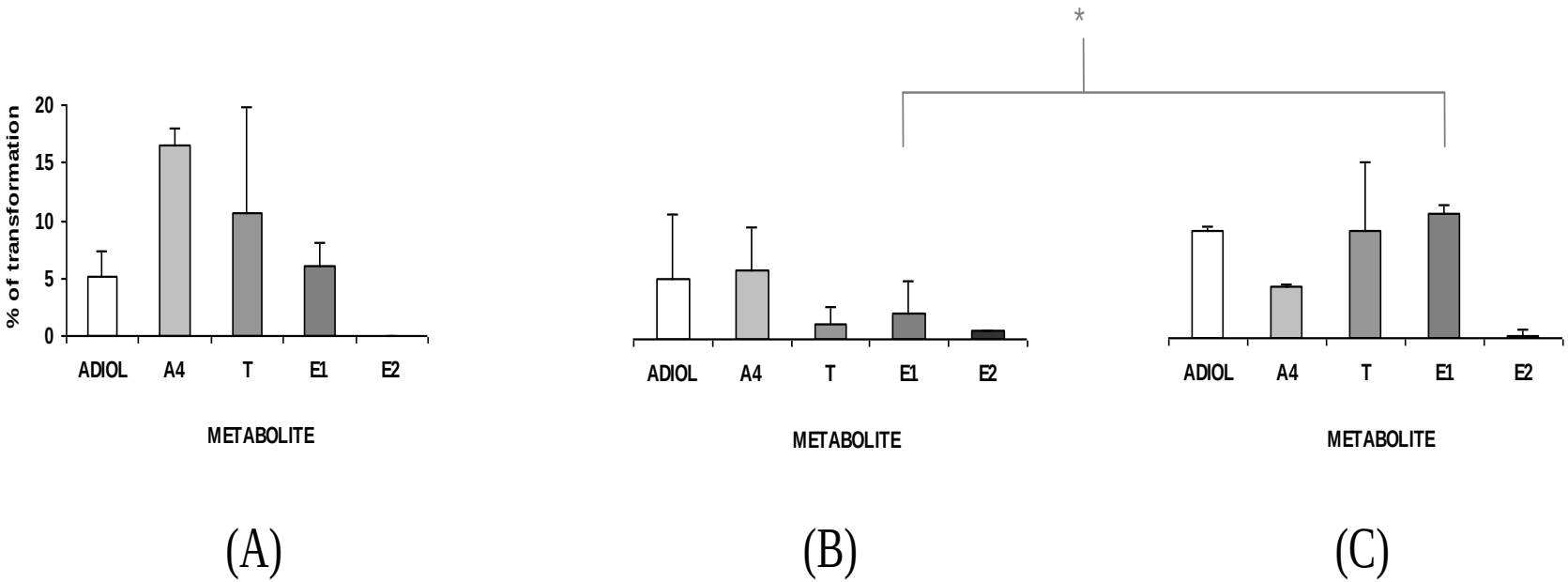
A4
E1
E2
METABOLITE

(A) (B) (C)

Figure 4


% of transformation
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METABOLITE
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METABOLITE

(A) (B) (C)